UNIVERSIDAD AUTÓNOMA DE MADRID
FACULTAD DE CIENCIAS

TRANSCRIPTIONAL CONTROL OF *ICP0* AND ITS EFFECTS ON HERPES SIMPLEX VIRUS-1 REPLICATION

HENA KHALIQUE

DEPARTAMENTO DE BIOLOGÍA MOLECULAR
UNIVERSIDAD AUTÓNOMA DE MADRID
MADRID, ESPAÑA

2015

TRANSCRIPTIONAL CONTROL OF *ICP0* AND ITS EFFECTS ON HERPES SIMPLEX VIRUS-1 REPLICATION

A thesis submitted to the Universidad Autónoma de Madrid for the degree of Doctor of Philosophy

Dr. Filip Lim, PhD
Director

Hena Khalique
PhD Student

Department of Molecular Biology
Faculty of Science
Autonomous University of Madrid
Madrid, Spain

2015

UNIVERSIDAD AUTÓNOMA DE MADRID
FACULTAD DE CIENCIAS

Filip Lim, **PhD**, Associate Professor at the Department of Molecular Biology of the Autonomous University of Madrid,

DECLARES,

that **Hena Khalique, MSc**, has carried out at the Department of Molecular Biology of the Autonomous University of Madrid and under his supervision, the original research entitled: "**Transcriptional control of *ICP0* and its effects on Herpes Simplex Virus-1 replication**", to obtain the Degree of Doctor of Philosophy (PhD).

Having reviewed the present work, I agree to its dissertation and defence.

Madrid, April 20, 2015

Filip Lim

UNIVERSIDAD AUTÓNOMA DE MADRID
FACULTAD DE CIENCIAS

Filip Lim, **PhD**, profesor titular del Departamento de Biología Molecular de la Facultad de Ciencias de la Universidad Autónoma de Madrid,

HACE CONSTAR,

Que **Hena Khalique** ha realizada bajo su dirección y con el máximo aprovechamiento, el trabajo titulado: "**Control transcripciónal de *ICP0* y sus efectos sobre la replicación del Virus Herpes Simplex tipo I**", para optar al Grado de Doctor.

Revisado el presente trabajo de investigación, quedo conforme con su presentación para ser juzgado.

Y para que así conste y surta los efectos oportunos, lo firmo en Madrid, a 20 de Abril de dos mil quince.

Filip Lim

To

Ma, Papa

& Kashif

ACKNOWLEDGEMENT

As I am finishing my thesis, I humbly take this opportunity to express my deep gratitude to all those wonderful people who made my entire journey extremely memorable and delightful.

At the outset, I would like to thank my thesis supervisor Dr Filip Lim for believing in me and giving me the opportunity to not only work on this project, but also make it my own. He gave me all the freedom I needed to carry out my project and implement my ideas, but was always there to keep me from going off track. His understanding, encouragement and personal guidance has helped me in all my research work and also in penning this thesis. He has not only helped me in developing my scientific thinking, but also encouraged to think out of the box. This five-year journey with him has been a great learning experience for me and will surely help me in all my future endeavours.

I gratefully acknowledge MAEC-AECID pre-doctoral scholarship for financial support during my PhD training.

I have had the pleasure of working alongside a great bunch of colleagues in the lab, and want to thank all of them. Vega - life in a foreign country would not have been so pleasant without your company. Thank you for helping me in my difficult times of settling in a foreign land. Alicia - it was a delight troubleshooting failed experiments and standardizing the recombination technique in the lab with you. Maria - you have always been a wonderful support for me and it was always a pleasure to share my thoughts with you. Thank you for boosting my morale every time I needed and for being a wonderful translator with doctors, government officials, bank clerks and many more. Predes, Antonio, Guillermo - I consider myself very lucky to have met you people here. You all left a deep mark in my life. I learned a lot from you and will miss you people the most. I will always cherish our lunchtime chats which always added more flavor to the meal. Alba, Hernan, Zulma - I owe so many of my smiles to you guys.

I owe a great deal to my Degree and Master students - Aurelio, Diana, Andrea, Jorge. I had a great experience working with you guys. You all taught me more than I taught you.

Special thanks to all my best friends whom I call my extended family – Priyanka, for being the most awesome person on the face of planet Earth ever; Tribhuwan, who was just a phone call away, and someone with whom I hung out and laughed the most; Dhruba, for being one of my ‘coolest’ friends who I always called for advice be it related to fashion, food or discussions over lame soap-operas; Pavan - for always being so supportive.

Sarfaraz, Rajiv, Preeti, Chetan - I will always cherish all our Christmas escapes to European countries. Smriti, Partha, Ananya, Raheema- Thank you for all the get-together parties and wonderful times.

I express sincere thanks to my parents for all the love and endless support they have given me all these years and for teaching me that anything is possible. Papa, Ma - I am nothing without you. You nurtured me with your love and blessings and made me what I am today. A heartfelt thanks to my sister and brothers for providing an extremely loving environment for me. My nephews and niece whom I fondly named “Chillar Party” - thank you for always bringing a smile on my face. A special thanks to my very considerate and extremely supportive in-laws.

I am grateful to almighty God - You have blessed me always and showed me the right path.

Finally, I want to thank a person who has been with me with unending, unconditional love; through thick and thin - my beloved husband and soulmate Kashif. Thank you for being with me, whenever I needed you the most. Thanks for being my rock, my pillar of strength. Thank you for having faith in me when I had the least in myself. Thanks for always being patient and nice to me and helping me believe in dreams, most importantly to conquer them.

Besides, several people, whose names may have been left out, contributed to make this experience an enriching one and I extend my sincere gratitude to all of them.

Hena

SUMMARY

Herpes simplex virus-1 (HSV-1) is one of the most common infectious agents found in humans, although only about a third of infected individuals exhibit symptoms such as cold sores and facial lesions. Its enveloped virions contain a 152 kilobases (kb) linear, double stranded (ds) DNA genome. HSV-1 replicates profusely in epithelial cells but in neurons, enters into latency, from which it can be reactivated to lytic replication by different external cues. The mechanism by which HSV-1 switches between these two life cycle modes is not known. An understanding of this will not only improve understanding of viral physiology but also enhance development of improved tools for viral vector-mediated gene therapy. During productive replication HSV-1 synthesizes approximately 75 proteins out of which five are translated immediately after infection. These are the **i**mmediate **e**arly (IE) proteins known as ICP0, ICP4, ICP22, ICP27 and ICP47 (ICP = infected cell protein). ICP0 is the first translated viral product and possesses multiple functions for the efficient progression of productive lytic infection. This thesis aimed to contribute to the understanding of the HSV-1 lytic-latent transition by testing if artificial control over *ICP0* transcription can be used to regulate HSV-1 replication.

The *ICP0* gene is duplicated in the HSV-1 genome; the internal repeat (IR) and terminal repeat (TR) regions each encode one copy. In the present study, I removed one copy of *ICP0* by deleting the IR region after which components of the Tetracycline-inducible system were inserted into the remaining copy to impose artificial transcriptional control of the gene. The IR-deleted mutant constructed using BAC technology confirms that the IR deletion does not affect viral replication in Vero cells. My results also indicate that the remaining copy of the *ICP0* gene in TR becomes a critical determinant of productive infection when a second gene in this repeat, that of *ICP34.5*, is additionally deleted. My study further investigates the ability to regulate viral replication through the consensus TAATGARAT element which has a key role in ICP0 promoter function and reports that distal displacement of the TAATGARAT element away from its natural position diminishes immediate-early ICP0 expression but results in surprisingly high late protein accumulation, altered protein cleavage and cytoplasmic translocation, all of which result in impaired viral growth. These defects could be reduced by repositioning the TAATGARAT motif to decrease the displacement but not by binding of tetracycline-responsive regulators to the artificially inserted tetO sites. The results further suggest that the HSV-1 proteins ICP0 and ICP4 can interfere with the tetracycline-responsive system and thereby disrupt the regulation. The study in this thesis supports the role of ICP0 as a critical switch component in HSV-1 productive infection but shows that high levels of ICP0 do not necessarily assure viral growth and may even be detrimental when expression kinetics are altered. Not only simulatory, but also inhibitory effects are mediated by the ICP0 promoter TAATGARAT element to impose complex dynamic control on ICP0 expression, and this cannot be reproduced by simply tethering VP16 to the promoter such as with the tetracycline-responsive system. Most

importantly, the present study indicates that: (1) simple ON and OFF switching of *ICP0* transcription does not result in efficient viral replication which is dependent on correct ICP0 kinetics and (2) the tetracycline-responsive system may not accurately control *ICP0* transcription due to interference by the ICP0 protein itself.

PRESENTACIÓN

El virus herpes simplex tipo I (VHS-1) es uno de los agentes infecciosos más comunes en el hombre, aunque sólo producen síntomas leves, como erupciones labiales y lesiones faciales, en un tercio de la población infectada. La estructura del virión está caracterizada principalmente por un genoma de DNA bicatenario lineal de 152 kilobases, y una envuelta lipídica. Tras la infección, en una primera fase lítica, el VHS-1 replica en células epiteliales hasta alcanzar las neuronas, donde establece un estado de latencia del que puede ser reactivado por diferentes estímulos externos. Dicho mecanismo de reactivación, a día de hoy desconocido, se propone como la clave no solo para esclarecer la fisiología del VHS-1 sino para el desarrollo de futuras herramientas, basadas en vectores virales, para terapia génica. Durante su replicación productiva, el VHS-1 sintetiza aproximadamente 75 proteínas, cinco de cuales son traducidas inmediatamente después de la infección. Dichas proteínas, denominadas proteínas inmediatamente tempranas (IE), son ICP4, ICP22, ICP27, ICP47 y ICP0 (ICP= acrónimo en inglés que significa "proteínas de célula infectadas"); ésta última es el primer producto viral traducido tras la infección y está dotada de múltiples funciones clave para el progreso eficiente de la fase lítica de la infección. El objetivo general de la tesis aquí presentada ha sido intentar esclarecer el mecanismo de inactivación y reactivación del VHS-1 mediante el control artificial de la transcripción de *ICP0* y su posible utilidad para la regulación de la replicación viral.

El gen de *ICP0* se encuentra duplicado en el genoma del VHS-1, estando una de las copias codificada por la repetición interna del genoma viral (IR) y la otra por la repetición terminal (TR). En este estudio se ha deleccionado la IR, eliminando así la copia del gen de *ICP0* presente en ésta, y se ha insertado un sistema inducible por tetraciclina para posibilitar un control artificial de la transcripción de la copia de ICP0 de la región TR. De esta manera se generó el mutante de delección en la IR mediante el uso de la tecnología BAC (acrónimo en inglés que significa "cromasoma bacteriano artificial"), confirmando que la ausencia de esta copia de ICP0 no afecta la replicación viral en células Vero. En dicho mutante se observó que la copia restante del gen de ICP0 en IR adquiere un papel determinante en la infección viral productiva cuando también se delecciona *ICP34.5*, presente en IR. Así mismo, este estudio investiga la capacidad de regular la replicación viral a través del elemento consenso "TAATGARAT", el cual posee una función clave en el promotor de ICP0, y pone de manifiesto que el desplazamiento de la posición del elemento TAATGARAT en el promotor disminuye la expresión inmediatamente temprana de ICP0; sin embargo, produce una sorprendente acumulación tardía de la proteína. Además se observa también un procesamiento proteico alterado así como una translocación citoplasmática, todo ello dando lugar a una disminución de la replicación viral. Dichos efectos se pueden revertir, al menos parcialmente, disminuyendo la distancia del desplazamiento de la posición del elemento "TAATGARAT"; sin embargo no se observa ninguna reversión como resultado de la unión de proteínas reguladoras inducibles por tetraciclina a los sitios tetO que se insertaron artificialmente. Por otra parte, los resultados sugieren

que las proteínas de VHS-1, ICP0 e ICP4, podrían estar interfiriendo con los componentes del promotor mínimo del sistema inducible por tetraciclina (tetO/CMV), bloqueando de este modo la regulación dependiente de tetraciclina.

El estudio presentado en esta tesis pone de manifiesto el rol de ICP0 como un componente crítico para la regulación de la infección productiva del VHS-1; no obstante, se ha observado que niveles altos de dicha proteína no aseguran necesariamente la replicación viral, llegando incluso a actuar en detrimento de ésta cuando su cinética de expresión se encuentra alterada. El elemento "TAATGARAT" del promotor de ICP0 no sólo media efectos activadores sino también inhibitorios para imponer un control complejo y dinámico de la expresión de ICP0. Estas funciones no se pueden reproducir a través de la mera unión de VP16 al promotor, como en el caso del sistema inducible por tetraciclina.

En resumen, el estudio indica que: (1) un simple interruptor para la transcripción de *ICP0* no es suficiente para una replicación viral eficiente, ya que depende de la correcta cinética de expresión de ICP0, y (2) la expresión de ICP0 interfiere con la regulación de la transcripción del sistema inducible por tetraciclina.

Index

FIGURES and TABLES

ABBREVIATIONS

AAV	Adeno-associated virus	**HDAC**	Histone deacetylase
AD	Alzheimer's disease	**hDaxx**	Human death domain-associated protein 6
ATRX	Alpha thalassemia/mental retardation syndrome X-linked	**HSV**	Herpes simplex virus
BAC	Bacterial artificial chromosome	**HVEM**	Herpes viral entry mediator
BHK	Baby hamster kidney	**ICP**	Infected cell polypeptide
cDNA	Complementary DNA	**IE**	Immediate early
CMV	Cytomegalovirus	**IF**	Immunofluorescence
CNS	Central nervous system	**IFN**	Interferon
CoREST	Corepressor of repressor element-1 silencing transcription	**IPTG**	Isopropyl β-D-1-thiogalactopyranoside
CPE	Cytopathic effects	**IR**	Internal repeat
C-terminal	Carboxy-terminal	**IRES**	Internal ribosome entry site
DAB	3, 3'-Diaminobenzidine	**kb**	Kilobases
DAPI	4', 6-diamidino-2-phenylindole	**kDa**	Kilodalton
DNA	Deoxyribonucleic acid	**KRAB**	Krüppel associated box
dNTPs	Deoxyribonucleotide triphosphates	**L**	Late
Dox	Doxycycline	**lacZ**	β-galactosidase
dsDNA	Double stranded DNA	**LB**	Lysogeny Broth
DTT	Dithiothreitol	**LAT**	Latency associated transcript
E	Early	**LV**	Lentivirus
E. coli	*Escherichia coli*	**miRNA**	microRNA
EDTA	Ethylenediaminetetraacetic acid	**MOI**	Multiplicity of infection
EF1α	Elongation factor 1α	**mRNA**	Messenger RNA
EGTA	Ethylene glycol tetraacetic acid	**NF-κB**	Nuclear factor kappa-light-chain-enhancer of activated B cells
EHV-1	Equine herpes virus-1	**N-terminal**	Amino-terminal
FBS	Fetal bovine serum	**Oct-1**	Octamer binding protein-1
GFP	Green fluorescent protein	**OD**	Optical density
HCF	Host cell factor	**PAGE**	Polyacrylamide gel electrophoresis
HCMV	Human cytomegalovirus	**PBS**	Phosphate buffered saline

PCR	Polymerase chain reaction	**ssDNA**	Single stranded DNA
PFA	Paraformaldehye	**TAE**	Tris-acetate EDTA
Pfu	Plaque forming units	**TBST**	Tris-buffered saline with Tween-20
PKR	dsRNA-dependent protein kinase	**TEMED**	Tetramethylethylenediamine
PP1	Protein phosphatase 1	**tetO**	Tetracycline operator
RCC1	Regulator of chromatin condensation-1	**tetR**	Tetracycline repressor
REST	Repressor element-1 silencing transcription factor	**TNF**	Tumor necrosis factor
RING	Really interesting new gene	**TSS**	Transcriptional start site
RNA	Ribonucleic acid	**tTA**	Tetracycline transactivator
RNA Pol	RNA polymerase	**tTS**	Tetracycline transsilencer
RT-PCR	Reverse transcription PCR	**TR**	Terminal repeat
rtTA	reverse tetracycline-responsive transactivator	**TRE**	Tetracycline responsive element
SDS	Sodium dodecyl sulfate	**UV**	Ultraviolet
SDS-PAGE	SDS-Polyacrylamide gel electrophoresis	**VP16**	Virion protein 16
SOB	Super optimal broth	**VZV**	Varicella zoster virus
SOC	Super optimal broth with catabolite repression	**WT**	Wild-type HSV-1
Sp100	Speckled protein of 100 kDa	**X-gal**	5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside

Introduction

1. INTRODUCTION

Viruses have role in eukaryotic evolution by mediating horizontal gene transfer (reviewed in (Keeling & Palmer, 2008)). Researchers have utilized this property in developing viral vectors as a tool for highly efficient nucleic acid delivery to specific cell types. Many vectors derived from viruses like lentiviruses, retroviruses, adenoviruses and adeno-associated viruses (AAV) have been generated for use in gene therapy (reviewed in (Robbins et al, 1998)). However, use of these vectors is often restricted due to low cargo capacity, possible genetic mutations due to integration into the infected cell genome, or possible elimination of the infected cells due to host immune responses.

HSV-1 is among the largest DNA viruses investigated for gene transfer (reviewed in (Glorioso, 2014)) and has been engineered as a vector for application in gene therapy (reviewed in (Lim, 2013)). The advantageous properties of HSV-1 as a vector are (1) natural neurotropism, (2) ability to exist as an episome in host cells, (3) large transgene capacity (it can package up to 160 kb of DNA), (4) high infectivity and (5) persistence in neurons in a latent state. Due to its ability to replicate vigorously in dividing cells and establish latency in neurons, HSV-1 has been considered ideal for oncolytic virotherapy ((Yao et al, 2010), reviewed in (Varghese & Rabkin, 2002) (Everts & van der Poel, 2005)) and gene therapy for neurological diseases such as Parkinson's and Alzheimer's disease ((Lilley & Coffin, 2003; Perez et al, 2004), reviewed in (Fink et al, 2000; Frampton et al, 2005; Marconi et al, 2010)). In addition, the ability of HSV-1 to efficiently infect a number of different cell types, such as muscle and liver, make it an excellent vector for treating non-neurological diseases. However, one of the drawbacks of HSV-1 vectors has been its complexity due to the large size of its genome. HSV-1 vector engineering has greatly advanced recently due to the availability of complete viral genome sequences and bacterial artificial chromosome (BAC) clones of some widely-used laboratory HSV-1 strains (Gierasch et al, 2006; Saeki et al, 1998; Tanaka et al, 2003) together with molecular tools such as mutagenesis by homologous recombination (Zhang et al, 1998) and Cre recombinase-mediated BAC retrofitting (Kim et al, 1998).

The ability of HSV-1 to switch between lytic infection and latency is an attractive property to exploit for HSV-1 vector engineering. On the one hand, efficient lytic growth in producer cells is required to generate high titers of vectors, while a latent-like state of the viral genome is desirable in target cells to ensure that the vector is transparent to the host. Although intensely investigated, the mechanisms by which the virus establishes and maintains latency or is reactivated into lytic replication are still not well understood. Knowledge of how HSV-1 switches between these two states will help not only in understanding the basic physiology of this virus but can also be applied to development of vectors as therapeutic tools. This understanding will also be helpful in the generation of easy-to-use packaging systems capable of producing high-titer homogeneous HSV-1 vectors.

The present study analyses the possibility of controlling the lytic-latent transition by manipulating HSV-1 regulatory genes.

1.1. Herpes Simplex Virus-1 (HSV-1)

HSV-1 (Lowenstein, 1919) belongs to *Herpesviridae*; a family of double stranded (ds) DNA viruses. The family name originates from the ancient Greek word *"Herpein"* meaning "to crawl" or "to creep", to describe spreading lesions and recurrent infections. Until now, around 90 viral species have been identified for *Herpesviridae*, which appear to be ubiquitous in the animal kingdom. The members of the *Herpesviridae* family are characterized by large genome size (up to 250 kilobases (kb)), wide host range and the ability to enter into a latent state in specific cell types (reviewed in (Roizmann et al, 1992)). The family has been further classified into three subfamilies: *Alphaherpesvirinae, Betaherpesvirinae* and *Gammaherpesvirinae,* based on their speed of replication, the cell type harboring latent virus and genomic analysis. HSV-1 belongs to the fast-growing, neurotropic *Alphaherpesvirinae*.

1.1.1. HSV-1 involvement in diseases

HSV-1 is ubiquitous in humans and on average 90% of the adult population is seropositive for anti-HSV-1 antibodies. Transmission of infection occurs either by direct mucosal contact or through abraded skin. HSV-1 is normally associated with oral, perioral, genital, skin and mucous membrane lesions. Recurrent HSV-1 infection in the eye may result in corneal scarring, sight threatening retinitis and blindness. Rarely, HSV-1 can spread to the central nervous system (CNS) and cause viral encephalitis (reviewed in (Liesegang, 2001)). Previous studies have pointed to a possible role of HSV-1 in Alzheimer's disease (AD) (reviewed in (Itzhaki & Wozniak, 2008)) proposing that viral infection acts in combination with genetic factors to provoke accumulation of the AD hallmark proteins β-amyloid (Aβ) and abnormally phosphorylated tau (P-tau). A recent study has shown that with the use of anti-viral compounds the level of Aβ and P-tau decreases in HSV-1 infected Vero cells (Wozniak et al, 2011).

1.1.2. Structure of the virion

The HSV-1 virion is a sphere of approximately 180 nm diameter and possesses a dsDNA genome (reviewed in (Liesegang, 1992)). The morphological organization of the mature virion can be divided into four parts: (1) an electron dense core; containing a DNA genome of 152 kb and a molecular weight of approximately 100 x 10^6 Dalton (Da) (Becker et al, 1968); (2) an icosahedral capsid; which protects the genome and consists of many viral proteins; (3) the tegument; an amorphous layer which surrounds the capsid and contains 20 different virus-encoded proteins with structural and regulatory roles; and (4) the envelope; an outer phospholipid bilayer. The intactness of

the envelope is an absolute requirement for the viability of the virion. The envelope is adorned with 12-13 virally encoded glycoproteins which help the virus to interact with target cells (**Figure 1A**).

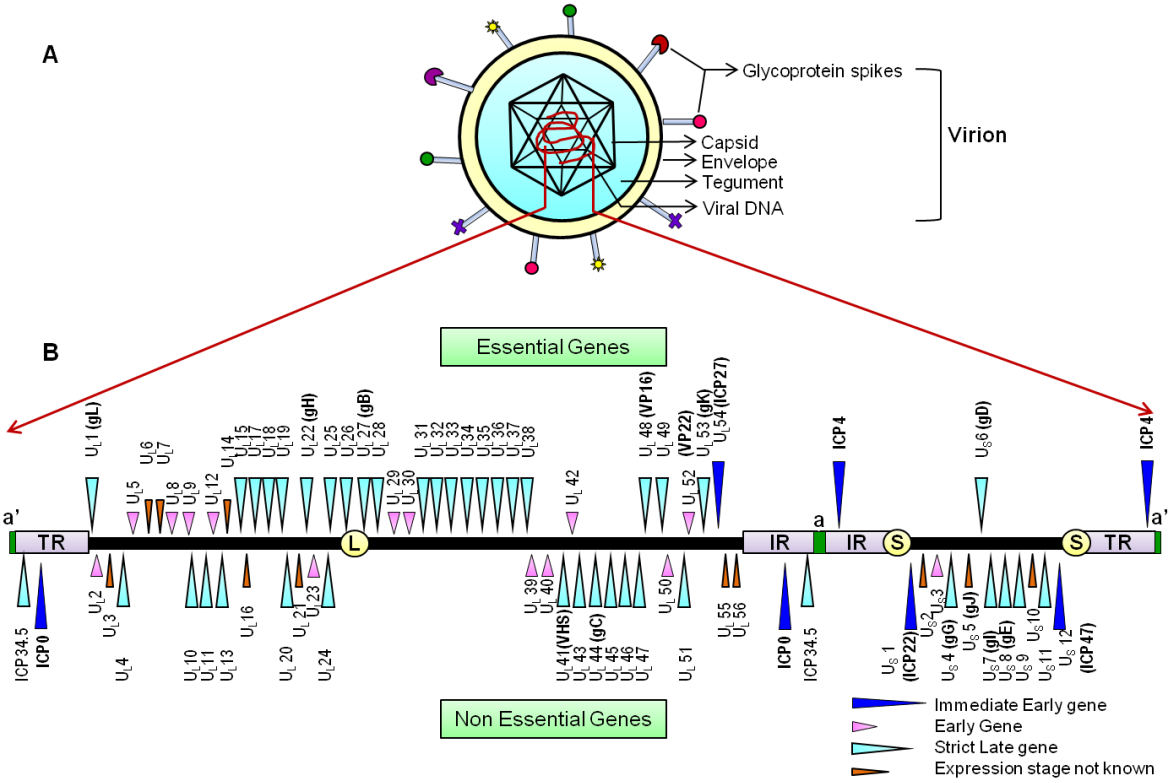

Figure 1. Schematic representation of HSV-1 genome.

A. Schematic representation of the mature HSV-1 virion consists of a dsDNA genome, an icosahedral capsid, the tegument and double layered envelope adorned with glycoprotein spikes.

B. Schematic representation of the 152 kb long dsDNA genome of wild type HSV-1. The unique long (U_L) and the unique short (U_S) segments are shown flanked by the internal repeat (IR) and the terminal repeat (TR). The unique origin of replication oriL is present in the U_L segment (L enclosed by circle) and two copies of oriS (S enclosed by circle) are present flanking the U_S segment. Two packaging signals (a and a') are present which are necessary for the packaging of the viral DNA into the virion. The 80 genes expressed in productive infection are indicated as essential or nonessential based on their requirement *in vitro*. Transcription of the viral genes occurs in three temporal phases after infection; immediate early (blue triangles), early (pink triangles) and late (turquoise triangles). The transcription stage of a few HSV-1 genes is not known and these are indicated as brown triangles.

1.1.3. The HSV-1 genome

The HSV-1 genome is a linear, 152 kb long dsDNA. It consists of: (1) two covalently linked unique fragments, one long (U_L) and one short (Us) which comprise 82% and 18% of viral DNA respectively; (2) flanking repeated sequences, terminal repeat (TR) and internal repeat (IR) which encode immediate early regulatory proteins and a late protein ICP34.5; (3) three *cis*-acting origins of DNA replication, one in the middle of the U_L region (oriL) and two flanking the U_S region (oriS); (4)

two packaging "a" sequences, one in the middle of the IR region and another at the end of the linear genome TR region (**Figure 1B**). The "a" sequences contain sites necessary for the packaging of viral DNA into the virion. The "a" sequence of the TR region is asymmetrically partitioned and consists of single base 3' extensions. These serve as cohesive ends to circularize and generate an "endless" intact genome (Mocarski & Roizman, 1982) by homologous recombination mediated by cellular factors (reviewed in (Boehmer & Nimonkar, 2003)). An alternative mechanism for circularization, especially in light of the fact that certain herpesviruses lack terminal repeats, involves direct ligation of genomic termini. The covalently linked U_L and U_S components can invert relative to each other to generate four genomic isoforms at equimolar levels (reviewed in (Roizman, 1979a; Roizman, 1979b)) which are all functionally equivalent and capable of independent replication (Jenkins & Roizman, 1986).

Complete sequencing of the HSV-1 genome has been carried out on many different isolates (Macdonald et al, 2012a; Macdonald et al, 2012b; McGeoch et al, 1988; Norberg et al, 2011; Szpara et al, 2010) revealing that the virus encodes about 80 genes. Approximately half of the genes are dispensable for *in vitro* viral replication, but play significant roles *in vivo* in virus-host interactions (**Figure 1B**) (reviewed in (Nishiyama, 1996; Ward & Roizman, 1994)).

1.1.4. Biology of HSV-1

HSV-1 exhibits two modes of life cycle: Lytic and Latent. HSV-1 is highly replicative in rapidly dividing epithelial cells but establishes life-long latency in neuronal cell bodies following retrograde transport from nerve terminals (Kristensson et al, 1971). The lytic cycle of HSV-1 begins in epithelial cells of the skin or mucosa following attachment of the virion to the cell surface. HSV-1 entry into target cells generally takes place either by fusion of the virion envelope with the plasma membrane or through endosomes. Depending on cell type, fusion can be pH-independent or may require low pH e.g HSV-1 entry into Vero cells is pH-independent (Koyama & Uchida, 1987) but requires a low pH for HeLa cells (Nicola et al, 2003). Viral entry is a complex process and involves interaction of multiple viral glycoproteins and cell surface receptors. Briefly, it takes place in two steps: (1) attachment of the viral particle to the cell surface, and (2) fusion of the viral envelope and plasma membrane.

Of the 12 or more glycoproteins present on the HSV-1 viral envelope, coordinated action of five glycoproteins: gC, gD, gB and the heterodimer gH and gL, is required for viral entry into the target cell. gC and gB independently interact with cell surface heparan sulphate proteoglycan and mediate initial viral binding (**Figure 2**). In the absence of both gB and gC, virus binding to the cell surface is severely reduced (Herold et al, 1994). gD is the main receptor-binding glycoprotein and binds to three classes of cell receptor: (1) herpes viral entry mediator (HVEM); a member of the TNF receptor family; (2) nectin 1 and nectin 2; members of the immunoglobulin superfamily and (3) 3-O sulphated heparan sulphate ((Montgomery et al, 1996; Shukla et al, 1999) reviewed in (Spear et al,

2000)). The binding of gD to one of these receptors initiates the conformational changes mediated by gB, gD, gH, and gL and triggers the fusion between the virion envelope and the plasma membrane (**Figure 2**) ((Fuller & Lee, 1992), reviewed in (Connolly et al, 2011; Reske et al, 2007; Spear & Longnecker, 2003)).

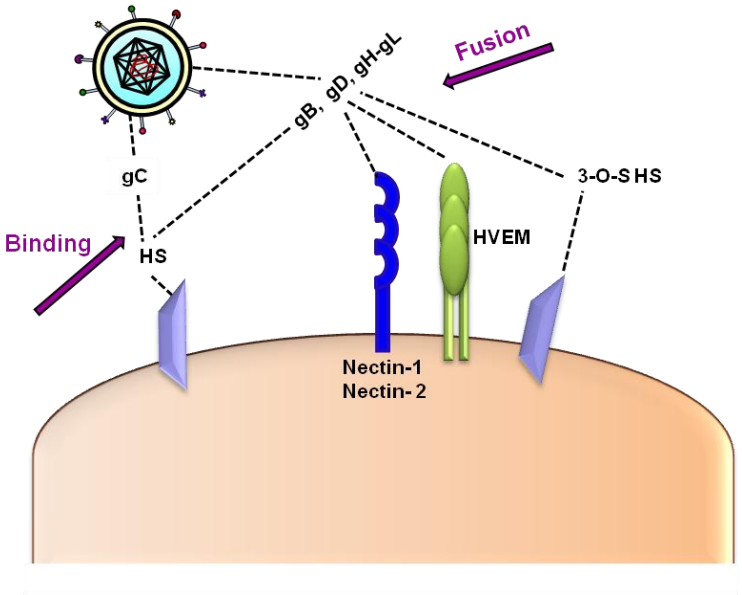

Figure 2. HSV-1 attachment and entry into the cell.

Initial attachment of the HSV-1 particle to the host cell surface is mediated through binding of the envelope glycoproteins gC and/or gB to cell surface heparan sulphate (HS) proteoglycan. Binding of the virus to the cell surface is followed by attachment of the envelope glycoprotein gD to cell surface receptors herpes viral entry mediator (HVEM), nectin 1/2 or 3-O sulphated heparan sulphate (3-O-S HS) which initiates the conformational changes mediated by gB, gD, gH and gL leading to the fusion of viral envelope with the cell plasma membrane.

After entry, the nucleocapsid is transported via the host's cytoskeleton elements to the nuclear pores where the genome is then introduced into the nucleus. A viral tegument protein (U_L 41) rapidly inhibits host protein synthesis and allows translation of viral transcripts by host ribosomes. Viral progeny of HSV-1 are produced by lytic replication. Virions which during the course of infection gain access to sensory nerve terminals undergo retrograde transport from the nerve endings in peripheral tissue to neuronal cell bodies deep in the spinal cord where HSV-1 establishes life-long latency (**Figure 3**) (reviewed in (Roizman & Whitley, 2013)). Periodic reactivation of the latent genome can occur spontaneously or in response to stimuli like ultraviolet (UV) light exposure, high temperature, fever, mental or physical trauma to the host, etc (Sawtell & Thompson, 1992; Wagner et al, 1975), bringing infectious viral progeny back to the nerve endings through anterograde transport, provoking skin lesions or mucosal ulcers (**Figure 3**) (reviewed in (Whitley et al, 1998)).

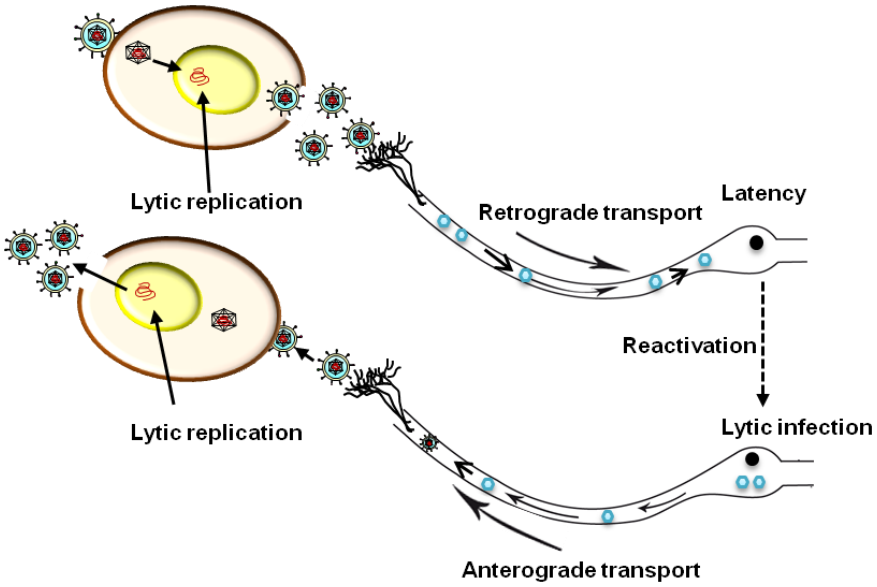

Figure 3. HSV-1 life cycle.

HSV-1 entry into an epithelial cell is mediated through fusion of the viral envelope with the plasma membrane and release of the nucleocapsid and tegument into the cytoplasm. Transport of the nucleocapsid to the nucleus is mediated by the host cytoskeleton and the genome is injected into the nucleus through nuclear pores. Following lytic replication in the infected epithelia, progeny virions are retrogradely transported to the cell bodies of sensory neurons innervating the infection site. Upon arrival to the cell body, the virus either initiates lytic expression or enters latency. Periodic reactivation from latency can occur spontaneously or in response to a variety of stimuli, including stress to the cell, high temperature or ultraviolet light exposure, resulting in the production of progeny virions that are anterogradely transported back to the nerve terminal to infect new epithelial cells.

1.1.5 HSV-1 lytic infection

HSV-1 gene expression during lytic infection is tightly regulated. In lytic growth, the 80 odd genes are expressed and categorized in three sets on the basis of their requirements for expression: immediate early (IE) or α genes, early (E) or β genes and late (L) or γ genes. These three classes are expressed in a temporal cascade (Honess & Roizman, 1974). Expression of IE genes is independent of *de novo* viral protein synthesis but strongly stimulated by virion protein 16 (VP16), a component of the viral tegument (Gaffney et al, 1985). The synthesis of IE polypeptides reaches a peak between 2 to 4 hours post infection (Honess & Roizman, 1974). IE genes transactivate early (E) gene transcription which results in the synthesis of E polypeptides with a peak of expression between 5 to 7 hours post infection (Honess & Roizman, 1974). E polypeptides include enzymes and viral DNA binding proteins necessary for replication. L genes are transcribed only after the completion of DNA replication and their expression is stimulated by the products of both earlier classes of genes (IE and E). The L polypeptides reach peak synthesis between 12 to 15 hours post infection (Honess & Roizman, 1974) and include the structural polypeptides of the virion.

Expression of IE genes is initiated by the tegument protein VP16 which is a 490 amino acid protein with a conserved core region and a C-terminal activation domain. The core of VP16 is divided into structured and unstructured regions (Liu et al, 1999). The structured region recognizes the TAATGARAT motif in DNA whereas the unstructured region interacts with cellular factors - octamer binding protein-1 (Oct-1) and host cell factor (HCF) to form the VP16 induced complex (VIC) (**Figure 4**) (reviewed in (Wysocka & Herr, 2003)). The formation of VIC facilitates the interaction of the activation domain of VP16 with host transcription factors and assists in the recruitment of host RNA polymerase II (RNA pol II) transcription machinery into the close proximity of IE promoters to initiate transcription (Gerster & Roeder, 1988; Klemm et al, 1995; O'Hare et al, 1988).

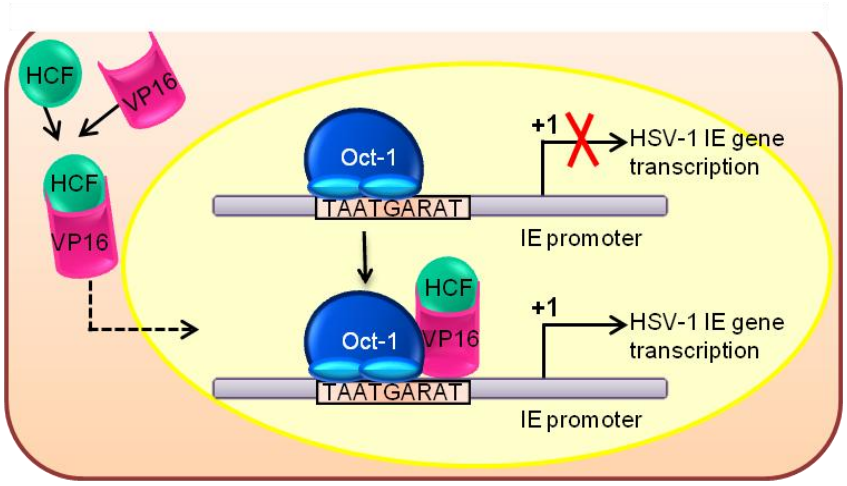

Figure 4. The VP16 induced complex (VIC)

VP16 has a C-terminal activation domain and transactivates transcription by binding to the TAATGARAT element (where R is a purine) present upstream of the TATA box in all IE gene promoters. VP16 forms a complex with the host cell factor (HCF) and localizes to the nucleus, where it binds to the host transcription factor octamer binding protein-1 (Oct-1) to form the VP16 induced complex (VIC). The binding of the VIC to the consensus TAATGARAT site enables the activator domain of VP16 to interact with host transcription factors and assist in the recruitment of the host RNA polymerase II transcription machinery into close proximity to IE promoters to initiate IE gene transcription.

HSV-1 expresses five IE genes which are designated as infected cell polypeptides (ICPs): ICP4, ICP0, ICP22, ICP27 and ICP47 (Honess & Roizman, 1974). **Figure 5** summarizes the regulation of the HSV-1 gene expression cascade during productive infection.

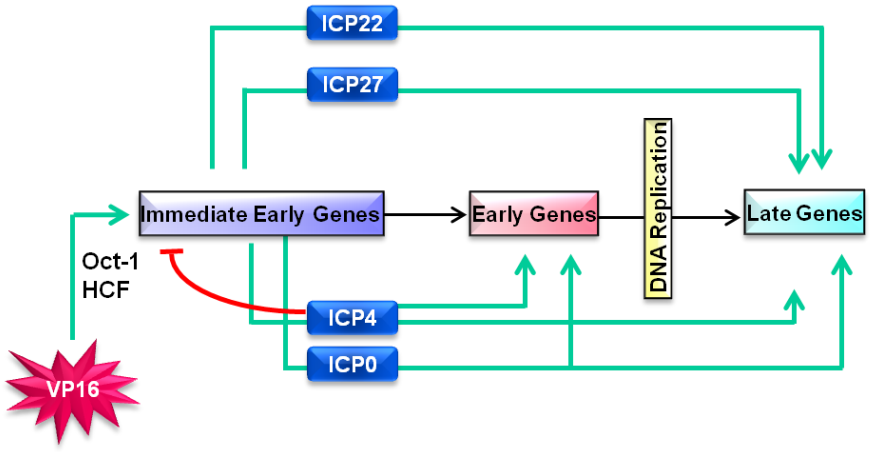

Figure 5. HSV-1 gene expression and regulation

Schematic diagram depicting HSV-1 gene expression cascades during productive infection. VP16 interacts with Oct-1 and HCF to transactivate immediate early gene transcription. The immediate-early gene products stimulate early gene expression which encodes functions necessary for replication of the viral genome. Late genes are expressed after the commencement of DNA replication. Immediate-early gene products regulate the coordinated expression of the HSV-1 genome. ICP4 and ICP27 are necessary for viral replication and transactivate the expression of early and late genes. ICP0 is a promiscuous transactivator of viral genes and in synergy with ICP4 transactivates early and late genes. ICP4 is also a repressor of *ICP0* and its own transcription. Although ICP22 is dispensable for viral replication in some cell types, it enables the optimal expression of late genes.

1.1.5.1. ICP0

ICP0 is a 775 amino acid multifunctional protein containing a RING (really interesting new gene) finger domain. It is the first translated product of HSV-1 after infection in a host cell and plays critical roles in overcoming host cell defenses, viral gene transactivation for efficient lytic replication, and reactivation from latency (Cai et al, 1993; Cai & Schaffer, 1992; Cai & Schaffer, 1989; Everett, 1984; Harris et al, 1989; Kawaguchi et al, 1997; O'Hare & Hayward, 1985) (reviewed in (Everett, 2000; Hagglund & Roizman, 2004)). The dependence of viral replication on ICP0 diminishes with increasing multiplicity of infection (MOI) and this IE protein is dispensable at high MOI (Everett, 1989; Sacks & Schaffer, 1987; Stow & Stow, 1986). At low MOI in the absence of functional ICP0, cellular repressors silence HSV-1 mRNA synthesis, favoring the entry of the virus into latency (reviewed in (Everett, 2000)). ICP0 plays a central role in HSV-1 biology due to its multiple functions such as E3 ubiquitin ligase activity (Boutell et al, 2002), counteraction of host anti-viral (interferon) responses (Mossman et al, 2000; Mossman & Smiley, 2002), degradation of nuclear domain 10 (ND10) bodies and inhibition of cellular repressors binding to the HSV-1 genome (Everett et al, 1998; Everett et al, 2008; Everett et al, 2006).

In the following sections I have briefly discussed ICP0 functions during HSV-1 infection to pose the question of whether manipulation of the gene encoding this protein can be a key to regulate HSV-1 lytic-latency balance.

1.1.5.2. Functions of ICP0

The HSV-1 genome possesses two copies of the *ICP0* gene, present in the IR and the TR regions. The *ICP0* gene is comprised of three exons encoding codons 1-19, 20-241 and 242-775 respectively (Perry et al, 1986).

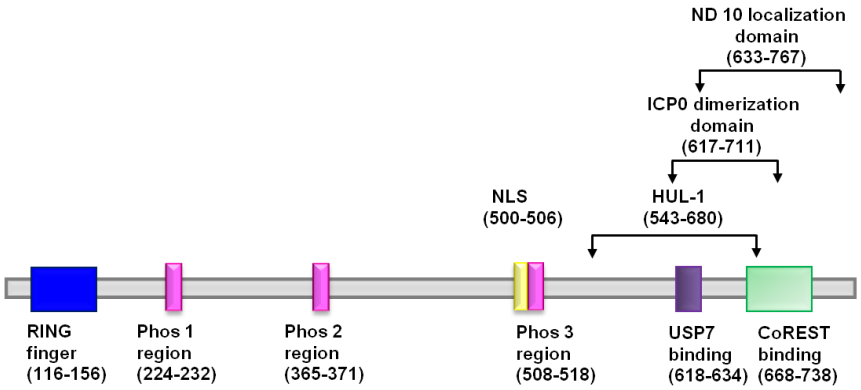

Figure 6. The functional domains of ICP0

The functional domains of ICP0 include the N-terminal RING finger motif (residues 116-156), regions of phosphorylation (Phos 1-3) (residues 224-232, residues 365-371, and residues 508-518), a C-terminal nuclear localizing signal (NLS) (residues 500-506), the Herpesvirus ubiquitin ligase (HUL) domain (residues 543-680), the ND10 localization domain (residues 633-767), a dimerization/multimerization motif (residues 617-711), the ubiquitin specific protease 7 (USP7) binding domain (residue 618-634) and the corepressor of repressor element-1 silencing transcription (CoREST) binding domain (residue 668-738).

Functional domains have been mapped in ICP0 (**Figure 6**) which carry out the functions described in the following subsections.

1.1.5.2.1. E3 ubiquitin ligase activity

ICP0 exhibits E3 ubiquitin ligase activity and targets proteins for degradation. In concert with E2 ubiquitin enzyme, ICP0 interacts with components of the ubiquitin proteasome pathway and attaches polyubiquitin chains to target proteins in a RING-finger-dependent manner (reviewed in (Smith et al, 2011)). ICP0 targets include numerous cellular proteins such as DNA-dependent protein kinase, the PML (promyelocytic leukemia) protein in ND10 bodies, Sp100 (speckled protein of 100 kDa), the DNA repair proteins RNF8 and RNF168, p53, and NF-κB (reviewed in (Smith et al, 2011)).

The C-terminus of ICP0 consists of another ubiquitin ligase domain (residues 543-680) known as herpesvirus ubiquitin ligase 1 (HUL1) ((Hagglund & Roizman, 2002), reviewed in (Hagglund & Roizman, 2004)). The activity of this domain is independent of the RING finger domain

(Hagglund & Roizman, 2002; Hagglund & Roizman, 2003; Hagglund et al, 2002) and specifically targets degradation of the E2 ubiquitin-conjugating enzyme cdc34 (Van Sant et al, 2001). However, a later study indicated that HSV-1 infection did not alter cdc34 protein levels (Everett, 2004). So far, no other target protein has been identified for HUL1.

The ICP0 orthologs of other herpesviruses like Bovine herpesvirus (BHV), Equine herpesvirus (EHV) and Varicella zoster virus (VZV) exhibit similar E3 ubiquitin ligase activity *in vitro* (Everett et al, 2010).

1.1.5.2.2. HSV-1 gene transactivation

ICP0 is a promiscuous transactivator of viral and non-viral genes in transient transfection and enhances viral replication both *in vitro* and *in vivo* (Cai & Schaffer, 1989; Everett, 1984; Everett, 1985; Gelman & Silverstein, 1985; Harris et al, 1989; Kalamvoki & Roizman, 2010; Sacks & Schaffer, 1987). The transactivational property of ICP0 functions through the RING finger domain, mutation of which renders ICP0 defective (Chen & Silverstein, 1992). ICP0 transactivates all three classes of HSV-1 genes i.e. IE, E and L (Cai & Schaffer, 1992; Mavromara-Nazos et al, 1986) independently or in synergy with ICP4 (Everett, 1984; Gelman & Silverstein, 1986). ICP0 does not *per se* recruit transcription factors to the DNA but modifies chromatin to provide a suitable substrate for the transcription machinery (Kalamvoki & Roizman, 2010).

1.1.5.2.3. Suppression of silencing of viral DNA

An important contribution of ICP0 in productive infection is the suppression of host intrinsic and innate defense machinery to prevent silencing of the viral genome. ND10 bodies are small nuclear structures involved in cellular processes like proliferation and differentiation but also in intrinsic resistance against RNA and DNA viruses (reviewed in (Everett & Chelbi-Alix, 2007)). ICP0 inhibits ND10 bodies by colocalizing and degrading them in a RING finger dependent manner (reviewed in (Everett, 2001; Everett & Chelbi-Alix, 2007)).

PML is an organizing protein of ND10 bodies. Previous studies have shown the potential role of PML in mediation of anti-viral functions (reviewed in (Regad & Chelbi-Alix, 2001)). A study on PML+/+ murine cells infected with an ICP0 mutant virus demonstrated profound inhibition of viral protein accumulation when treated with interferon (IFN)-alpha (α) or IFN-gamma (γ). The inhibition was higher when these IFNs were used in combination. However, similar experiments with wild type (WT) virus in PML+/+ cells or with the ICP0 mutant or WT virus in PML-/- cells showed minimal IFN inhibitory effect on viral protein accumulation (Chee et al, 2003), suggesting that the anti-HSV-1 state induced by exogenous IFN is mediated by PML (Chee et al, 2003). ICP0 induces proteasome-dependent degradation of PML, especially SUMOylated forms of the protein (Boutell et al, 2003; Everett et al, 1998; Muller & Dejean, 1999). An enhancement in growth and plaque formation was observed in the ICP0 null mutant when grown in a cell line artificially depleted for PML bodies

(Everett et al, 2008; Everett et al, 2006). The mechanism by which PML might act on intrinsic anti-viral defense mechanisms is yet to be discovered but other relevant ICP0 targets involved are other ND10 components contributing to intrinsic resistance: Sp100, hDaxx (human death domain-associated protein 6) and ATRX (alpha thalassemia/mental retardation syndrome X-linked) (Everett et al, 2008; Lukashchuk & Everett, 2010).

1.1.5.2.4. Suppression of chromatinization of the viral genome

Another model of cellular intrinsic resistance proposes rapid chromatinization of viral DNA upon infection by cellular histone proteins through the activities of histone deacetylase (HDAC) enzymes. To initiate IE transcription HSV-1 recruits lysine specific demethylase-1 (LSD-1) to the IE promoters to demethylate repressive histone protein H3 Lys9 (H3K9) (Liang et al, 2009). LSD-1 is a part of a cellular repressor complex which includes other components like histone deacetylase 1 (HDAC1), histone deacytylase 2 (HDAC2), BRAF35 and corepressor of repressor element-1 silencing transcription (CoREST). LSD-1 is stabilized by CoREST (Shi et al, 2005; Yang et al, 2006) which in turn needs repressor element-1 silencing transcription factor (REST) for stabilization. Knock down of REST or CoREST affects LSD-1 stability and availability for binding to the IE promoter, eventually affecting IE mRNA accumulation. LSD-1 mediated demethylation of histone proteins initiates viral IE transcription and translation. Translated ICP0 then binds to CoREST and displaces HDAC1 and HDAC2 from the repressor complex paving the way for E and L gene transcription (reviewed in (Roizman, 2011)).

1.1.5.2.5. Counteraction of anti-viral response

Interferons (IFNs) are cytokines with anti-viral and immunomodulatory functions (reviewed in (Le Page et al, 2000; Pestka et al, 1987; Sen, 2001)). IFN-α or IFN-beta (β) are secreted by cells as an innate response to viral infection whereas IFN-γ is an unrelated protein, produced almost exclusively by activated NK cells and T lymphocytes. IFNs activate IFN-stimulated genes (ISGs) which collectively function to inhibit viral replication and spread. ISGs can also be activated by viral infection in the absence of IFN (Wathelet et al, 1992) through interferon regulatory factors (IRFs); a family of transcription factors with broad range of functions (reviewed in (Mamane et al, 1999)). The role of ICP0 in counteraction of both IFN-dependent and -independent antiviral responses has been deduced from studies which show; (1) hypersensitivity of ICP0 mutants to IFN with decreased levels of viral mRNA transcripts and severely reduced plaque formation ability (Harle et al, 2002; Mossman et al, 2000); (2) enhancement in the growth of ICP0 mutants (up to 1000-fold) in IFN- α/β/γ null mutated mice (Leib et al, 1999) and (3) blocking of IRF3- and IRF7-mediated cellular responses by ICP0 RING finger dependent activity (Lin et al, 2004).

1.1.6. HSV-1 latency

HSV-1 latency can be defined as retention of a complete viral genome as a non replicative episomal molecule in a host cell without production of any infectious virions (reviewed in (Efstathiou & Preston, 2005)). After productive infection, HSV-1 may contact sensory neurons innervating the site of primary infection and migrate to their neuronal cell bodies in trigeminal ganglia where the expression of all viral genes is repressed with the exception of latency associated transcripts (LATs) (Stevens et al, 1987), leading to the establishment of latency. Since its discovery in 1987 (Stevens et al, 1987) LATs have been constantly studied for their involvement in the lytic / latency / reactivation cycle of HSV-1. The general conclusion from this research is that LATs are not essential for latency establishment or maintenance, nor for reactivation to the lytic phase (reviewed in (Efstathiou & Preston, 2005)). Functional properties assigned to LATs are- (1) participation in establishment of latency; (2) protection of the host cell from virus-induced apoptosis; (3) modulation of viral IE gene expression; and (4) participation in mechanisms of *in vivo* virulence (reviewed in (David C. Bloom, 2011)).

HSV-1 can remain in a quiescent latent state in neurons throughout the lifespan of the host or may reactivate occasionally to the lytic form followed by anterograde transport to innervated peripheral sites (**Figure 3**) (reviewed in (Efstathiou & Preston, 2005)). The molecular events underlying the establishment and maintenance of HSV-1 latency are complex as host neurons can survive virus-induced cytopathic effects and maintain latency until reactivated due to external cues.

1.1.6.1. Role of ICP0 in HSV-1 latency and reactivation

The possible roles of ICP0 in viral latency come from previous studies where ICP0 mutants enter a quiescent state when infected at low MOI (Sacks & Schaffer, 1987; Stow & Stow, 1986). These mutant genomes can remain quiescent for many days and can be reactivated by superinfection with human cytomegalovirus (HCMV) or VZV (Stow & Stow, 1989). The reactivation function of ICP0 maps to exon 2 of *ICP0* as mutants deleted for this region fail to reactivate (Harris et al, 1989). More detailed studies with ICP0 functional domains indicate involvement of the RING finger motif in removal of repressive chromatin and activation of quiescent genomes (Ferenczy et al, 2011). In an *in vivo* study Sawtell et al concluded that ICP0 is not essential as a component of the switch from latency to lytic-phase, but rather that it is critical in the initiation of the lytic cycle and production of infectious progeny once exit from latency has been accomplished (Thompson & Sawtell, 2006). In a recent study a neuron-specific host microRNA (miRNA) was identified which directly targets ICP0 to promote HSV-1 latency (Pan et al, 2014).

Taken together, the above findings suggest that ICP0 plays critical roles in viral replication, latency and reactivation and therefore manipulation of ICP0 expression might be a key to switch HSV-1 between its two life modes: lytic replication and latency.

1.1.6.2. Other factors with a role in latency

The HSV-1 mutant *in*1814 contains an insertion mutation in the coding sequence of VP16, impairing IE transactivation due to failure of the mutant VP16 protein to form a complex with cellular factors and the viral promoter element TAATGARAT. When used to infect non-complementing cells, the *in*1814 mutant enters a quiescent state in culture (Jamieson et al, 1995) and establishes latency *in vivo* (Steiner et al, 1990). The replication defect of *in*1814 can be partially overcome by expressing ICP0 in cells prior to the infection (Ace et al, 1989; Steiner et al, 1990). These observations have led to the hypothesis that the replication block in this mutant occurs before or at the IE gene expression stage. These studies indicate VP16 as an important contributor of viral replication, the absence of which pushes the mutant into a quiescent state. However, in one study Sears et al observed insignificant effects of VP16 on reactivation of a latent virus and concluded that lack of VP16 alone cannot account for latency establishment (Sears et al, 1991).

1.2. Inducible systems for gene expression

The ideal inducible system to regulate gene expression should include features such as: (1) tight regulation with no basal gene expression in the uninduced state; (2) high levels of gene expression in the presence of the inducer; (3) full reversibility of induction; (4) recapitulation of effects observed *in vitro* in models *in vivo* and (5) non-toxic and non-interfering inducers.

Many inducible regulatory systems for mammalian gene expression have been developed, employing a variety of inducers such as heavy metals (Brinster et al, 1982; Mayo et al, 1982; Searle et al, 1985), steroid hormones (Hynes et al, 1981; Israel & Kaufman, 1989; Lee et al, 1981), heat shock (Wurm et al, 1986), antibiotics (Gossen & Bujard, 1992) and other small molecule ligands such as Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Baim et al, 1991). However, some of the above mentioned systems are unsuitable for certain applications due to inducer toxicity. Furthermore, undesirable pleiotropic effects have been observed in some cases. e.g. in the estrogen-based inducible system a chimeric molecule of the estrogen receptor dimerizes in response to the inducer estrogen and binds to galactose-responsive transcription factor 4 (GAL4)-responsive promoters, leading to the activation of target gene expression (Braselmann et al, 1993). A major drawback of this system is that endogenous genes containing similar sequence elements are also modulated. In addition, endogenous levels of estrogen may also activate basal transgene expression, resulting in “leakiness” of the system.

1.2.1. Tetracycline-inducible system

The tetracycline (Tet) inducible gene expression system in eukaryotes was first reported in plants. Gatz et al utilized the regulatory elements of the Tn*10*-specific tetracycline resistance operon from *E. coli* to construct an inducible system to regulate the transcription from tetracycline operator (tetO) sequences inserted into the cauliflower mosaic virus (CaMV) 35S promoter (Gatz et al, 1992;

Gatz & Quail, 1988). In mammalian systems, Tet regulated gene expression was first achieved in 1992 by Bujard and co-workers to regulate the expression of the *Photinus pyralis* luciferase reporter gene (Gossen & Bujard, 1992). Kim et al in 1995 utilized this system to control β-glucuronidase gene (*uidA*) expression in a recombinant HCMV (Kim et al, 1995). Thereafter, many laboratories employed the Tet-inducible system to regulate gene expression in different viral vectors ((Corti et al, 1999; Haberman et al, 1998; Hofmann et al, 1996), reviewed in (Goverdhana et al, 2005)).

The Tet system consists of: (1) a minimal promoter (in the original version, derived from the CMV IE promoter) juxtaposed to seven repeats of tetR binding sites (tetO) and (2) a transcriptional transactivator known as the tetracycline-responsive transactivator (tTA). tTA is a fusion protein of the transactivating domain of the HSV-1 VP16 protein and the DNA-binding domain of the tetracycline repressor (tetR) of *E. coli* (Gossen & Bujard, 1992; Gossen et al, 1995). In the absence of the antibiotic tetracycline or its analogue doxycycline, tTA binds to the tetO sites and activates the transcription of the transgene whereas in the presence of the same antibiotic, tTA dissociates from the tetO sites and transactivation of the transgene ceases, allowing control over transgene expression by presence or absence of antibiotic in the medium (**Figure 7A**). Since the tTA protein has been observed to be toxic to mammalian cells (reviewed in (Gossen et al, 1993)), an autoregulatory system for tTA expression was developed. The gene encoding for tTA was also placed under control of the Tet inducible system to make the expression of tTA itself inducible. This autoregulatory system was observed to improve inducibility of gene expression compared to the constitutively expressing tTA system, both in cultured cells and in animal models (Shockett et al, 1995).

There are two basic variants of the Tet inducible expression system: the Tet-OFF system with tTA (Gossen & Bujard, 1992) and the Tet-ON system with reverse tetracycline responsive transactivator (rtTA) (Gossen et al, 1995). The rtTA protein differs from tTA by four amino acid changes in the original tetR domain. This mutation reverses the responsiveness of the rtTA protein to the inducer (tetracycline or doxycycline). In contrast to tTA, rtTA requires tetracycline/doxycycline for binding to tetO sites and is unable to bind in absence of tetracycline/doxycycline due to conformational changes (**Figure 7B**). In summary, Tet-OFF refers to a system where transgene is expressed in the absence of tetracycline/doxycycline whereas Tet-ON refers to a system where transgene is expressed in the presence of tetracycline/doxycycline. More recently, Urlinger et al have generated a novel mutant transactivator, rtTA2S-M2 which can regulate tetO controlled transgene expression with better inducibility and at tenfold less inducer concentration (Urlinger et al, 2000).

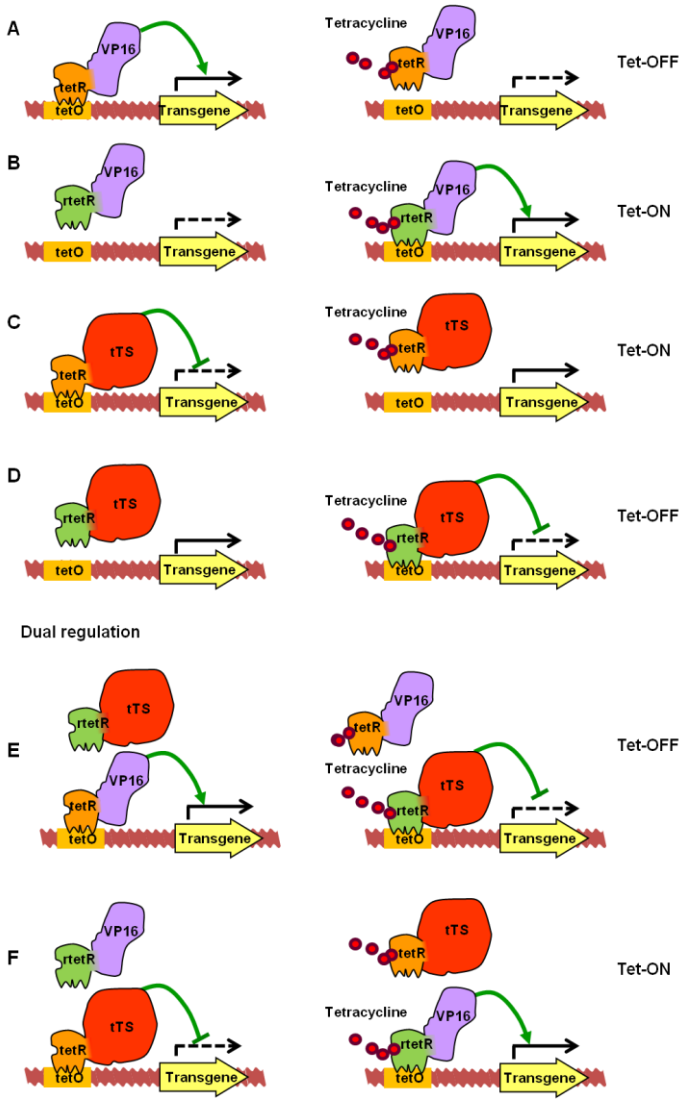

Figure 7. Regulation of gene expression by tetracycline transregulators.

A. Tetracycline controlled transactivator (tTA) system: tTA is a fusion protein of the DNA-binding domain of the tetracycline repressor (tetR) of *E. coli* and the transcriptional activation domain of HSV-1 virion protein 16 (VP16). In the absence of tetracycline, tTA binds to tetR binding sites in DNA (tetO) and activates transcription of the linked transgene. In the presence of tetracycline, tTA dissociates from tetO and terminates transcription of the transgene.

B. Reverse tetracycline responsive transactivator (rtTA) system: rtTA is a mutant version of the transactivator tTA with four amino acid changes in the tetR domain. In the presence of tetracycline, rtTA binds to tetO sites and activates transcription of the linked transgene. In the absence of tetracycline, rtTA dissociates from tetO and transcription of the transgene is absent.

C. Tetracycline responsive silencer protein (tTS) system; tTS is a fusion protein of tetR and transcriptional silencing domains of the Kid protein /or the Krüppel associated box (KRAB) domain of the human Kox1 zinc finger protein. In the absence of tetracycline, tTS binds to tetO sites and suppresses transcription of the linked transgene preventing leaky expression of the transgene. In the presence of tetracycline, tTS dissociates from tetO and transcriptional suppression of the transgene is absent.

D. Reverse tetracycline responsive silencer protein (rtTS) system: rtTS is a mutant version of the transcriptional silencer tTS with four amino acid changes in the tetR domain. In the presence of tetracycline, rtTS binds to tetO sites and suppresses transcription of the linked transgene whereas in the absence of tetracycline, rtTS dissociates from tetO and transcriptional suppression of the target gene is absent.

E and F. Combined use of Tetracycline transregulators (tTA, rtTS and rtTA, tTS). In the absence of tetracycline, rtTS dissociates from tetO whereas tTA binds tetO with high affinity and activates transcription and expression of the target gene. Response of the transcription with respect to the presence of tetracycline has been indicated on extreme right. This system is Tet-OFF, as the transcription of the transgene in this system occurs only in absence of tetracycline. In rtTA, tTS dual system, in the presence of tetracycline, tTS dissociates from tetO whereas rtTA binds tetO with high affinity and activates transcription and expression of the target gene. This is a Tet-ON system (as indicated on extreme right) as transcription of the transgene occurs only in the presence of the tetracycline.

To address the problem of leaky basal gene expression in Tet inducible systems, Bujard and coworkers generated a Tet regulatory silencer protein tTSKid; a fusion of tetR repressor with the transcriptional silencing domain of the Kid protein (Freundlieb et al, 1999). *In vitro* studies

demonstrated that tTSKid suppresses basal expression levels by 1000-fold without affecting maximal induced expression of the reporter transgene (Freundlieb et al, 1999). Later, Lamartina et al constructed a bicistronic vector containing a single regulatory cassette co-expressing both activator (rtTA2S-M2) and repressor (tTSKid) elements by inclusion of an internal ribosome entry site (IRES) (Lamartina et al, 2003). In the absence of tetracycline/doxycycline, tTSKid binds to the tetO sites and results in negligible transgene expression whereas in the presence of tetracycline, tTSKid binding to tetO sites is replaced with rtTA2S-M2 which induces a 1000-fold increase in transgene expression (Lamartina et al, 2003).

Another version of the Tet regulatory silencer was generated by Deuschle et al in which the Krüppel associated box (KRAB) domain of the human Kox1 zinc finger protein (Margolin et al, 1994) was fused to the *E. coli* tetR protein to create the transcriptional repressor tetR-KRAB (Deuschle et al, 1995). In the absence of tetracycline/doxycycline, tetR-KRAB binds to the tetO sites and exerts its silencing activity, blocking the expression of the reporter gene which is restored in the presence of tetracycline/doxycycline due to release of tetR-KRAB from the DNA (**Figure 7C**). In addition, a Tet-OFF version of tetR-KRAB has also been generated (rtTS-KRAB) in which reporter gene expression is silenced in the presence of tetracycline/doxycycline (**Figure 7D**) (Szulc et al, 2006). Current Tet-inducible systems with dual regulators: tTA and rtTS (**Figure 7E**) or rtTA, tTS (**Figure 7F**) have been generated to improve inducibility for this gene expression regulatory system.

Yao et al described another version of the Tet system (T-REx) (Yao et al, 1998) which differs from the conventional Gossen and Bujard Tet-system (Gossen & Bujard, 1992) in number and placement of tetO sites. The T-REx system contains two tetO repeats located 10 bp 3' to the CMV minimal promoter TATA box. Unlike the conventional Tet system, T-REx uses the whole tetR protein as repressor.

1.2.2. Tetracycline-inducible gene expression in HSV-1

Previous studies have reported successful use of the Tet-inducible system to regulate transgene expression in HSV-1 derived amplicon vectors. HSV-1 amplicons are plasmids bearing the expression cassette of gene of interest, the HSV-1 origin of replication and the HSV-1 packaging signal (Spaete & Frenkel, 1982). HSV-1 amplicons are packaged as concatemers of up to one viral genome length into HSV virions in the presence of a helper virus. Helper viruses are replication-defective HSV-1 viruses containing mutations or deletions within an essential IE gene, such as *ICP4* (Geller et al, 1990).

Ho et al incorporated the firefly *Photinus pyralis* luciferase reporter gene linked to the tetracycline-responsive promoter system into HSV-1 amplicon vectors and observed gene expression modulation both *in vitro* and *in vivo* (Ho et al, 1996). They observed 50-fold repression of gene expression in the presence of tetracycline which could be relieved, allowing maximal expression to be

reached within 10-12 hours after the removal of the antibiotic. In another Tet-OFF study, localized and high levels of β-galactosidase expression were observed in mice infected with an HSV-1 amplicon vector encoding the *Lac Z* gene and transactivator tTA. Transgene expression was observed to be reduced tenfold in the presence of doxycycline (Fotaki et al, 1997). In a later study, Schmeisser et al tested different promoters linked to the tetracycline-responsive element (TRE) in an HSV-1-based vector system to identify one with the maximum level of transgene induction. After testing constructs containing the TRE linked to: a minimal CMV promoter, or a minimal HSV-1 ICP0 promoter or a truncated HSV-1 ICP0 promoter with only one TAATGARAT motif, they reported the highest level of transgene expression using the truncated ICP0 promoter but the highest relative induction (with respect to the presence and absence of antibiotic) using the CMV minimum promoter (Schmeisser et al, 2002). Inducible transgene expression from this vector system was observed for several days in irreversibly differentiated NT2 cells (NT2 neurons) (Schmeisser et al, 2002).

Evidence for long-term transgene expression in HSV-1 vectors is provided by the study of Gao et al (Gao et al, 2006). They compared two vector designs: one where tTA and the *Lac Z* gene were expressed separately by tetO linked promoters and another where the *Lac Z* gene was expressed by a tetO linked promoter but a modified neurofilament heavy gene promoter was used to express transactivator tTA. The results showed that both vector designs supported inducible gene expression *in vitro* and *in vivo* for short term (4 days) but long-term (2 months) *in vivo* inducible expression was observed only from the vector expressing tTA from the modified heavy gene neurofilament promoter (Gao et al, 2006).

Yao et al demonstrated Tet-regulated expression of the *Lac Z* gene in replication-defective HSV-1 vectors using the T-REx system (Yao et al, 1998). They replaced both copies of the ICP0 coding sequence with gene sequences expressing tetR and the essential viral gene U_L9 with tetO-regulated *Lac Z* reporter gene and reported 300 to1000-fold tetracycline-regulated gene expression in different cell types (Vero, PC12 and NGF-differentiated PC12) (Yao et al, 2006). Similar efficient and regulated gene expression was observed *in vivo* following intracerebral and footpad inoculations in mice (Yao et al, 2006). Jiang et al in 2012 used this Tet-regulated HSV-1 vector to demonstrate a safe and efficacious method of vector delivery to rat brains (Jiang et al, 2012). They administered the Tet-inducible HSV-1 vector intranasally in rats and detected Tet-inducible β-galactosidase expression in cortical neurons, olfactory bulb, hippocampus, striatum, cortex, medulla, cerebellum, ventricles, and nasal septum (Jiang et al, 2012). Curiously, they observed that the intranasal viral administration method was less cytotoxic than intravenous or stereotactic delivery methods (Jiang et al, 2012).

1.2.3. Inducible control of HSV-1 Replication

Until now, very few attempts have been made to artificially regulate HSV-1 replication. Evidence for inducible HSV-1 replication comes from the study of Yao et al. They constructed the Tet

inducible HSV-1 recombinant KTR27 (Yao et al, 2010) by placing *ICP27* transcription under tetO control and replacing ICP0 coding sequences with gene sequences expressing tetR. They reported 1000 to 250,000-fold inducibility with respect to viral titer of KTR27 mutant in response to tetracycline. Intratumoral inoculation of this mutant markedly inhibited tumor growth in a xenograft model of human lung cancer in nude mice and this effect could be efficiently controlled by local co-delivery of tetracycline (Yao et al, 2010).

Using a different approach, Preston et al constructed the conditional mutant *in*1820K carrying mutations in the genes encoding ICP0, ICP4 and VP16. The mutant *in*1820K contains a host range mutation of the ICP0 promoter, a temperature-sensitive mutation in ICP4 and an insertion in the VP16 coding region which abolishes its IE gene transactivation function. Cultured cells could be infected by the *in*1820K mutant virus at an MOI of 5 without detectable cytopathology at the non-permissive temperature whereas the virus could undergo lytic replication to high titers in permissive conditions (Preston et al, 1997). These results indicate that VP16 manipulation could be an alternative approach to control viral replication by controlling the expression of all IE genes. However, since VP16 is a virion-associated structural protein, deletion of its gene may result in other effects on virus viability.

As mentioned earlier, HSV-1 circularizes its linear genome in host cells after infection either by direct ligation of the terminal ends (Davison & Wilkie, 1981; Mocarski & Roizman, 1982; Strang & Stow, 2005) or through homologous recombination between directly repeated sequences of the linear genome (Yao et al, 1997). This process employs the cellular protein regulator of chromatin condensation-1 (RCC1) (Umene & Nishimoto, 1996). Both circularization and viral DNA synthesis of WT HSV-1 in the tsBN2 cell line (a baby hamster kidney cell line (BHK) that carries a temperature-sensitive mutation in the gene encoding RCC1) were observed to be reduced but not abolished at the non-permissive temperature. When RCC1 was absent, negligible infectious WT progeny were detected (Strang & Stow, 2007), suggesting control of viral gene circularization as an alternative means to regulate viral replication.

HSV-1 replication is complex due to involvement of a large number of viral as well as cellular factors. Since HSV-1 has complicated gene expression cascades, a convincing reasonable approach would be to regulate the virus at a very early step of infection. ICP0 is the first translated product of HSV-1 and based upon the functions which this protein carries out (as discussed earlier in this section), regulation of ICP0 expression may be a viable strategy to impose control over HSV-1 replication. In the following section I have briefly discussed the basal and inducible activities of the ICP0 promoter, an understanding of which will be helpful in designing gene expression control systems.

1.3. ICP0 promoter elements

The regulatory elements present in the ICP0 core promoter regions are illustrated in **Figure 8**. These elements are: TATA box (T), Sp1 binding site (S), ICP4 binding site (4), G-box (G), CCAAT box (C), F2 (F) factor binding site, NF-κB-like binding site (NF), TAATGARAT (TG) and TAATGARAT-like (TL) motifs (Davido & Leib, 1998; Douville et al, 1995). Most of these regions act as protein factor binding sites and contribute towards the transcriptional regulation of the *ICP0* gene. Cellular factors like Sp1, CCAAT enhancer binding protein and F2 bind to the ICP0 promoter and activate ICP0 expression. The ICP4 binding site in the ICP0 promoter serves as a repressive motif at which ICP4 binds and represses *ICP0* transcription (Faber & Wilcox, 1986). The promoter region from -95 to -37 bp is important for constitutive ICP0 promoter activity in both neuronal and non-neuronal cells. The inducible activity of the promoter lies in the region from -550 to -129 bp. ICP0 promoter activity is regulated by both viral and non-viral factors.

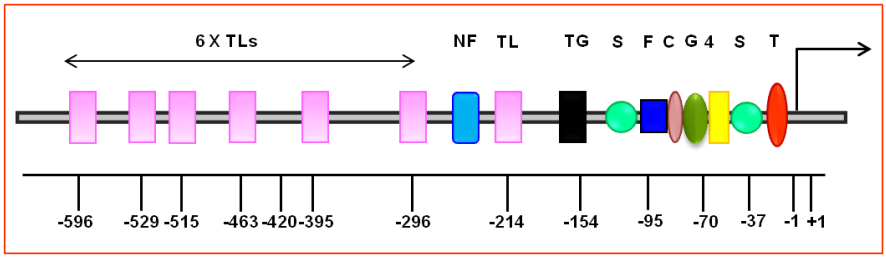

Figure 8. Schematic representation of the ICP0 promoter elements.

Map of the regulatory elements of the ICP0 promoter located upstream of the transcriptional start site (rightward pointed arrow). These elements are: TATA box (T), Sp1 binding site (S), ICP4 binding site (4), G-box (G), CCAAT box-binding factor site (C), F2 binding site for an unidentified factor (F), consensus TAATGARAT motif (TG), TAATGARAT like sites (TL) and NF-κB-like binding site (NF). The location of these elements is shown (not to scale) relative to the transcriptional start site (+1).

The ICP0 promoter has multiple TAATGARAT related motifs located at positions -576 to -154 bp from the transcriptional start site (TSS), but one of these containing the complete octamer sequence ATGCTAATGATAT (located at -154 bp from TSS) was identified as the most responsive motif (Kwun & Jang, 2000). VP16 along with the cellular factors Oct-1 and HCF forms the VP16 inducible complex (VIC) which binds to the complete TAATGARAT motif and after specific interactions with transcription factors strongly transactivates *ICP0* transcription (Kristie & Roizman, 1987; McKnight et al, 1987). The integrity of the ATGC portion is crucial for Oct-1/VP16 complex formation and mutation in this sequence results in loss of complex formation (Douville et al, 1995). Due to the presence of the overlapping octamer sequence (ATGCTAAT) other cellular octamer-binding proteins compete with Oct-1 for binding to the TAATGARAT motif and repress VP16-mediated *ICP0* transactivation (Douville et al, 1995; Kemp et al, 1990). Deletion of the

TAATGARAT motif removes the inhibitory effects of other Oct proteins (Oct-2.4 and Oct-2.5) on the ICP0 promoter (Thomas et al, 1998) suggesting the TAATGARAT motif as a mediator of both positive and negative effects of cellular transcription factors. Studies on VP16 show that its transactivation via VIC is short range and functions only from a promoter proximal position but can produce long range transactivation when fused to DNA-binding domains of other proteins (e.g. GAL4) (Hagmann et al, 1997). The inability of VP16 to transactivate from long distances could be a natural mechanism of the virus to ensure undesired long range effects on other viral promoters.

In nature HSV-1 can transit between replicative (lytic) and silent (latent) life modes. The study in this thesis was carried out to test if this transition can be simulated *in vitro* by artificially imposing control over HSV-1 replication. Since HSV-1 replication is complex and involves a large number of cellular and viral factors, the most likeable approach to regulation would be to target the earliest step of the replication. ICP0 is the first translated product of HSV-1 and performs central functions in viral replication, latency maintenance and reactivation to the lytic cycle (see section 1.1.5.2); therefore is an attractive candidate for manipulation to achieve the goal. In the present study I targeted *ICP0* transcription and generated mutations in the ICP0 promoter, to examine the possibility of producing an "OFF" state with no viral growth, which can be switched to an "ON" state with efficient lytic replication. Within the ICP0 promoter I targeted the consensus TAATGARAT motif positioned at -154 bp relative to the TSS to control VP16 mediated activation of the ICP0 promoter. The idea was to test if the artificial tetracycline inducible system can functionally substitute for natural VP16-mediated transactivation. Specifically, it was investigated if specific binding of Tet system regulators (rtTA, tetR-KRAB) to the tetO sites can inducibly regulate *ICP0* transcription and if this regulation can simulate control in viral replication. I observed that although deletion of the IR region from the HSV-1 genome does not significantly reduce viral growth in culture, this minimized genome became sensitive to additional mutations which have no phenotype in the wild-type genome: deletion of the *ICP34.5* gene and alteration of the ICP0 promoter TAATGARAT element. Furthermore, distal displacement of the TAATGARAT element unexpectedly augments ICP0 accumulation which considerably affects mutant replication. The present study indicates a strong dependence of productive infection on the complex kinetics of ICP0 processing, translocation and accumulation which cannot be mimicked by the binding of rtTA at tetO sites.

Objectives

2. OBJECTIVES

Amongst the five immediate-early (IE) genes of HSV-1, *ICP4* and *ICP27* are known to be essential for viral growth in culture, with regulatory functions in the viral lytic cycle (DeLuca et al, 1985; Dixon & Schaffer, 1980; Preston, 1979; Sacks et al, 1985; Watson & Clements, 1978) whereas *ICP22* and *ICP47* are non-essential *in vitro* (Longnecker & Roizman, 1986; Post & Roizman, 1981). The requirement for ICP0 in viral replication however has been observed to be MOI dependent: ICP0 is dispensable at high MOI but is essential at low multiplicity for viral gene expression and efficient progression of lytic infection. Analysis of an ICP0 null mutant (Stow & Stow, 1986) at the single cell level revealed a cell type-dependent MOI threshold above which the mutant virus replicates normally but below which it becomes quiescent (Everett et al, 2004; Sacks & Schaffer, 1987). Nonproductively infected cells can express viral proteins and mutant virus can enter either quiescence, produce stalled infections, or in some cases even initiate plaque formation (Everett et al, 2004; Sacks & Schaffer, 1987).

Considering the vital role of ICP0 in determining the fate of viral replication, the overall aim of this thesis was to test the hypothesis that productive HSV-1 lytic replication can be regulated by controlling *ICP0* transcription. My approach consisted of the following specific aims:

2.1. To verify if a single copy of repeat sequences is sufficient for HSV-1 replication *in vitro*

The HSV-1 genome has two copies of the IE genes - *ICP0* and *ICP4* which are present as direct and internal repeats. For my work on developing HSV-1 derived vectors, deletion of one set of these genes facilitates the unambiguous engineering of the other set. This can be achieved by homologous recombination in bacteria ((Muyrers et al, 1999; Zhang et al, 1998) reviewed in (Muyrers et al, 2001)). To test if a single copy of repeat sequences is sufficient for HSV-1 replication, I compared the *in vitro* titer of an HSV-1 mutant deleted for internal repeat sequences to that of the WT virus.

2.2. To determine if ICP0 expression can be regulated artificially

The tetracycline-inducible system (Gossen & Bujard, 1992) was utilized to regulate the transcription of the remaining copy of *ICP0* in the HSV-1 mutant deleted for internal repeat sequences. This insertion of tetracycline-responsive elements were combined with the deletion of ICP0 promoter regulatory elements to generate potentially conditional mutants. The control of ICP0 expression was assayed in cell lines expressing tetracycline transregulators in the presence or absence of doxycycline.

2.3. To determine the effect of ICP0 promoter mutations on ICP0 expression and HSV-1 replication

The effect of ICP0 promoter manipulations on HSV-1 replication was analyzed by measuring the titer and ICP0 expression level of the mutants in infected Vero cells and cell lines expressing Tet regulators (transcriptional transactivators and silencers).

Materials and Methods

3. MATERIALS

3.1. Reagents

Dulbecco's Modified Eagle Medium (DMEM), Opti-MEM and TrypLE were purchased from Gibco/Life Technologies, Paisley, UK. Antibiotics used in cell cultures and bacterial cultures (penicillin, streptomycin, ampicillin, kanamycin, tetracycline, chloramphenicol), L-arabinose and doxycycline hyclate (Dox) were purchased from Sigma-Aldrich (St. Louis, MO). Puromycin was obtained from Gibco/Life Technologies (Grand Island, NY). Fetal bovine serum (FBS, product number F7524) was from Sigma-Aldrich (St. Louis, MO) whereas TRIzol, Lipofectamine-LTX and Plus reagents were purchased from Life Technologies (Carlsbad, CA).

3.2. Bacterial strains

Strain	Genotype	Source/ Ref
DH10B	F^- mcrA Δ(*mrr-hsd*RMS-*mcr*BC) Φ80dlacZΔM15 ΔlacX74 *end*A1 *rec*A1 *deo*R Δ(*ara*,*leu*)7697 *ara*D139 *gal*U *gal*K *nup*G *rpsL* λ^-	(Durfee et al, 2008; Grant et al, 1990)
DH5α	F^- Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK^-, mK^+) *pho*A *sup*E44 λ^- *thi*$^-$1 *gyr*A96 *rel*A1	(Taylor et al, 1993)
PIR1	F^- Δ*lac169 rpoS(Am) robA1 creC510 hsdR514 endA recA1 uidA(ΔMluI)::pir-116*	Life Technologies (Carlsbad, CA), catalogue number-C1010-10

3.3. Cell lines

Cell line	Origin	Culture Media	Source/Ref of the plasmid used for construction of stable cell lines
Vero	African green monkey kidney epithelial cell	F10 medium: DMEM supplemented with 10% FBS, 100U/ml	ATCC* CRL 1586 (Earley EM, 1988)

		penicillin and 100 μg/ml of streptomycin	
Vero-tetRKRAB	Vero cell line expressing tetracycline repressor-KRAB fusion protein	F10 medium supplemented with puromycin (3 μg/ml)	pEFKRAB (see section 4.2.4.2)
Vero-rtTA	Vero cell line expressing tetracycline reverse transactivator (rtTA)	F10 medium supplemented with puromycin (3 μg/ml)	(Mak et al, 2010)
U2OS	Human osteosarcoma cell line	F10 medium	(Yao & Schaffer, 1995)

*ATCC is the registered trademark of the American Type Culture Collection.

3.4. Virus

The parental virus strain used in this study was strain F of HSV-1(Ejercito et al, 1968). The viral genome of strain F cloned in a bacterial artificial chromosome (BAC) plasmid (Tanaka et al, 2003) was used for the construction of all the mutants.

3.5. Plasmids

Plasmid	Selection marker	Source / Ref
pAXKS	Ampicillin	*Ase*I/ *Xho*I fragment from cos6 ligated into pBluescriptKSII(+)
pBluescriptKSII(+)	Ampicillin	(Alting-Mees & Short, 1989)
Cos6	Ampicillin	(Cunningham & Davison, 1993)
Cos48	Ampicillin	(Cunningham & Davison, 1993)
pCTP	Ampicillin	(Saeki et al, 2001)

pDRIVE-CAG	Zeocin	Invivogen, San Diego, CA
pEEHG	Ampicillin	(Wade-Martins et al, 2001)
pEF-IRESP	Ampicillin	(Hobbs et al, 1998)
pGEMT Easy	Ampicillin	Promega, A1360
pGZSpe	Ampicillin, Zeocin	PCR amplified *Sh ble* gene using oligonucleotides PACSPE and UL5Z cloned into pGEMT Easy vector
pI0prtTRK	Ampicillin	*Spe*I/*Nco*I pLVPRT-tTRKRAB and *Nco*I/*Eco*RI pAXKS ligated into *Spe*I/*Eco*RI pSKC481.9
pI0prtTRZ	Ampicillin, Zeocin	*Not*I/*Spe*I pGZSpe cloned into *Not*I/*Spe*I pI0prtTRK
pLD-puro-2A-rtTA-TcVA	Ampicillin, Puromycin	Addgene, Cambridge MA, (Mak et al, 2010)
pLOsG	Ampicillin	*Sal*I/*Spe*I pEEHG blunted with Klenow and religated in presence of dNTPs
pLOsG_Z	Zeocin	*Not*I/*Spe*I pGZSpe cloned into *Not*I/*Spe*I pLOsG
pLVPRT-tTRKRAB	Ampicillin	Addgene, Cambridge MA, (Szulc et al, 2006)
pRSVβgal	Ampicillin	(Bonnerot et al, 1987)
pSKC481.9	Ampicillin	*Xho*I/*Eco*RI Cos48 ligated into pBluescriptSK
pSK+Kana-RPSL	Ampicillin,	Addgene, Cambridge MA, (Wang et al,

	Kanamycin	2009)
pTetICP0B	Ampicillin	*Sac*II/ *Bam*HI fragment from Cos6 (Cunningham & Davison, 1993) ligated into pUHD10-3
pTGT0	Ampicillin, Kanamycin	*Xba*I/*Xho*I RP-Kan^R cassette (amplified using oligonucleotides TAAT and KANA3P) ligated into *Xba*I/*Xho*I pTetICP0B
pUG6SP-tTA	Ampicillin	Addgene, Cambridge MA, (Zilio et al, 2012)
pUHC13-3	Ampicillin	(Gossen & Bujard, 1992)
pUHD10-3	Ampicillin	Kind gift from Gossen, M, University of Heidelberg, Germany
p175	Ampicillin	(Perry et al, 1986)
pΔ34.5TGT0	Ampicillin, Kanamycin	*Xba*I/*Xho*I RP-Kan^R cassette (amplified using oligonucleotides ATN5 and KANA3P) ligated into *Xba*I/*Xho*I pTetICP0B
pΔ34.5T0	Ampicillin, Kanamycin	ligation of *Sac*I/*Bam*HI pAXKS (1.4 kb), *Xba*I/*Sac*I pΔ34.5TGT0 (1.76 kb) and *Xba*I/*Bam*HI/*Sac*I pΔ34.5TGT0 (2.6 kb) fragments

3.6. Oligonucleotides

The following oligonucleotides were synthesized by Isogen Life Science.

Name	Sense	Sequence (5'- 3')
AMP5'OUT	Backward	CCCACTCGTGCACCCAACTG

AMP3'OUT	Forward	CCCGTATCGTAGTTATCTAC
ATN5	Forward	*Xba*I TCTAGACCGAGACTAGCGAGTTAGACAGGC AAGCACTACTCGCCTCTGCACGCACATGTTA ATTAAGTTTTATGGACAGCAAGCGAACCGG *Pac*I
CMVTATAFW	Forward	ATAGAAGACACCGGGACCGA
C6B	Backward	AAGAACCCCATTAGCATG
C3DRK		CCCCGGGGACGGGGCCGCCCCTTAATTAAG TTTTATGGACAGCAAGCGAACTAAGGTCGG ACTCCGGATGGTGCCTCGAGTTTACCACTCC CTATCAGTG
C3DRK1	Forward	AATGAGTTTCTTCGGGAAGG
C3DRK2	Backward	AAAACAGCGTGGATGGCCGTC
ICP0TAfw	Forward	ATCACCACAGAAGCCCCGCCT
ICP0E2rv	Backward	TTCGGTCTCCGCCTCAGAGT
I34.53OUT	Forward	AGGCGGTCATCGGGCCGTG
I4sen	Forward	CGACACGGATCCACGACCC
I4ant	Backward	GATCCCCCTCCCGCGCTTCG
KANA 3P	Backward	*Xho*I Filler sequence* CTCGAGGCACCATCCGGAGTCCGACCTTAG CAATACCTTAATTAATCAGAAGAACTCGTC AAGAAGGCGAT *Pac*I

KRAB200	Forward	GCAAAAGTGAGTATGGTGCC
KRAB3P	Forward	TCCCATCGAATTCGAAGTTGAG
MARIZEO	Forward	CACTTTGTGGCAGAGGAGCAGGA
PACSPE	Backward	GACTAGTTAATTAAGTTGAAAAAAGGGGCC
RPREV	Backward	GGAGTGGTAGTATATACACGAG
SEQK5	Forward	CGTGCTTTACGGTATCGCCG
SU1LOXHSV	Forward	TGCAGGAATTCGATATCAAG
SU2LOXHSVUL4	Backward	CGCCCTGGAATACGCAGACA
TAAT	Forward	*Xba*I TCTAGAGCATGCTAATGATATTCTTTGGGGG CGCCGGGTTGGTCCCCGGGGACGGGGCCGC CCCTTAATTAAGTTTTATGGACAGCAAGCGA ACCGG *Pac*I
TetΔTGK3P	Backward	CCACCGCGGGGCGGCCCCGTCCCCGGGGAC CAACCCGGCGCCCCCAAAGAGATCTCTATC ACTGATAGGGAGATCTCTATCACTGATAGG GAGTCAGAAGAACTCGTCAAGAA
TN5PROM	Backward	TTCCCAACCTTACCAGAGGGCG
ΔTGR5P	Forward	TTCTTTGGGGGACACCGGGTTGGTCCCCCAA ATCGGGGGCCGGGCCGTGCTATGGACAGCA AGCGAACCGG
US3A	Backward	AAATGTCGGCCATCCAGAAAACGTCCCGGA GGACCACAGTGGCTTCCCCCCCAGTTACCA ATGCTTAATCAG

UL5A	Forward	TTATAACCCCGGGGGTCATTCCCAACGATCACATGCAATCTAACTGGCTCGCACTTTTCGGGGAAATGTG
UL5Z	Forward	CTCGAGTTATAACCCCGGGGGTCATTCCCAACGATCACATGCAATCTAACTGGCTCAATTAAATTTTTCAAAAGTAGTTGAC

*Filler sequence = 31 base-pair random DNA sequence inserted as a homologous arm for posterior deletion of the RP-Kan^R cassette in the Tet-inR mutant.

3.7. Antibodies

Primary	Secondary	Dilution/ Assay
Anti-ICP0 mouse monoclonal (Santa Cruz Biotechnology, Inc, CA)	anti-mouse IgG peroxidase conjugate (1:2000) (Sigma-Aldrich, St. Louis, MO), Alexa-Fluor 488 conjugated anti-mouse (1:500) (Molecular Probes/Life Technologies, Eugene, OR)	1:500, 1:50 Western, immunofluorescence (IF)
Anti-ICP4 mouse monoclonal (Abcam, Cambridge, UK)	anti-mouse IgG peroxidase conjugate (1:2000) (Sigma-Aldrich, St. Louis, MO)	1:2000 Western
Anti-GAPDH mouse monoclonal (Abcam, Cambridge, UK)	anti-mouse IgG peroxidase conjugate (1:2000) (Sigma-Aldrich, St. Louis, MO)	1:3000 Western
Anti-HSV-1 rabbit polyclonal (Dako Cytomation, Glostrup, Denmark)	anti-rabbit IgG peroxidase conjugate (1:2000) (Sigma-Aldrich, St. Louis, MO)	1:1000 Immunosubstrate staining
Anti-PML rabbit polyclonal (Santa Cruz Biotechnology, Inc, CA)	Alexa-Fluor 555 conjugated anti-rabbit (1:500) (Molecular Probes/Life Technologies,Eugene, OR)	1:50 IF

3.8. Buffers

The following standard buffers were used in the experiments throughout:

1X Tris-acetate (TAE)	40mM Tris, 20mM acetic acid, and 1mM EDTA
1X Phosphate buffered saline (PBS)	137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4 and 1.8mM KH_2PO_4
1X Electrophoresis buffer	25 mM Tris, 192 mM glycine, 0.1% SDS
1X Transfer buffer	48 mM Tris, 39 mM glycine, 10-20 % methanol
1X TBST(Tris-Buffered Saline with Tween 20)	50 mM Tris.HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20

4. METHODS

4.1. MOLECULAR BIOLOGY TECHNIQUES

4.1.1. Agarose gel electrophoresis

1% (w/v) agarose gels were prepared using 1X TAE buffer (see section 3.8). SYBR Safe (Life Technologies, Paisley, UK) DNA gel stain was added to the mixture at a final concentration of 1X. Loading buffer (10X: 1X TAE, 50% (v/v) glycerol, 0.025% bromophenol blue) was added to DNA samples prior to loading at a 0.1 volume. For DNA size markers a 10 kb DNA ladder (Thermo Scientific, Carlsbad, CA) was used in all gels. The bands were visualized on a Gel Logic 1500 Imaging System (Eastman Kodak, Rochester, NY).

4.1.2. Restriction enzyme digestions

Restriction enzyme digests were performed on plasmid DNA in a total volume of 30 µl containing 0.5 µg (plasmid) - 2 µg (BAC) DNA, 0.5 - 1 µl restriction enzyme (10 U/µl), 3 µl of the appropriate restriction buffer (10X) as specified by the manufacturer (Thermo Scientific, Carlsbad, CA) and double-distilled water. The digests were incubated at 37°C from 1 hour to overnight followed by electrophoresis on a 1% agarose gel. The bands were visualized on a Gel Logic 1500 Imaging System (Eastman Kodak, Rochester, NY).

4.1.3. Elution and purification of DNA from gel bands

A small hole was made in the bottom of a 0.5 ml microcentrifuge tube and was covered with glass wool. An agarose slice containing the DNA band of interest was excised using a clean scalpel and placed in the microcentrifuge tube on top of the glass wool. The tube containing the gel slice was then placed inside a larger (1.5 ml) microcentrifuge tube and centrifuged at 11,000 x*g* for 1 minute. The liquid obtained in the 1.5 ml microcentrifuge tube was collected and added to phenol at a ratio of 1:1. The liquids were mixed by vortexing and then centrifuged at 11,000 x*g* for 5 minutes to separate the aqueous and organic phases. The upper aqueous phase was carefully collected with a pipette, transferred to a fresh 1.5 ml microcentrifuge tube and DNA precipitated by the addition of 0.1 volumes 3M sodium acetate (pH 5.4), 3 volumes ethanol and 10 ng/µl of glycogen carrier. The tube was incubated at -20°C for 1 hour and then centrifuged at 11,000 x*g* for 30 minutes at 4°C. The DNA pellet obtained after centrifugation was washed once with 1 ml of 70% ethanol, air dried and dissolved in double-distilled water. The concentration of purified DNA was measured using a nanodrop spectrophotometer (ND-1000).

4.1.4. DNA ligation reactions

For each ligation reaction, 50 ng of vector DNA was mixed at a 1:3 molar ratio with the insert DNA along with 0.2 µl (5 U/µl) of T4 DNA ligase (Thermo Scientific, Carlsbad, CA) in ligase buffer

(Thermo Scientific, Carlsbad, CA) in a final volume of 10 µl. A control sample with no insert DNA was always included to reveal uncut or relegated vector. Reaction mixtures were incubated overnight at 16°C or 1 hr at room temperature.

4.1.5. Polymerase chain reaction (PCR)

For a 50 µl reaction, 1 µl of template DNA (1-50 ng) was added to a 0.2 ml PCR tube containing 10 µl GoTaq flexi buffer (5X), 4.8 µl 25 mM magnesium chloride ($MgCl_2$), 1 µl 10 mM dNTPs (dATP, dTTP, dCTP and dGTP) mixture, 1 µl of each 5 mM oligonucleotide and 0.2 µl GoTaq DNA polymerase (5 U/µl, Promega). The volume was made up to 50 µl using double-distilled water. The PCR mixture was incubated in an automated thermocycler running the following amplification program: one cycle of 95°C for 5 min, 30 cycles of (1) 94°C for 30 sec, (2) 55-65°C (depending on the T_m of the oligonucleotides used) for 30 sec, (3) 72°C for 1 minute (depending on the length of the PCR product; ≈1 minute/ kb), and 1 cycle of 72°C for 5 minutes. PCR products were resolved on a 1% agarose gel and bands were visualized on a Gel Logic 1500 Imaging System (Eastman Kodak, Rochester, NY).

4.1.6. RNA isolation and RT (reverse transcription)-PCR

Cells were harvested for total RNA using TRIzol reagent, according to the manufacturer's protocol. Briefly, cell monolayers were lysed in TRIzol reagent (1 mL TRIzol Reagent for a 35 mm culture dish) containing guanidinium thiocyanate. The cells were lysed directly in the culture dish by pipetting the mixture up and down several times and then collected into a 1.5 ml microcentrifuge tube. To extract RNA from the TRIzol preparation, 200 µl chloroform was added and the mixture was shaken vigorously by hand for 15 seconds. The mixture was incubated at room temperature for 3 minutes and then centrifuged at 12,000 x*g* for 15 minutes to separate the 3 layers; lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. The aqueous phase was collected carefully in a new 1.5 ml microcentrifuge tube and an equal volume of isopropanol was added. The mixture was incubated at room temperature for 3 minutes and then centrifuged at 12,000 x*g* for 10 minutes to yield an RNA pellet. The pellet was washed with 75% ethanol and then dried at room temperature to ensure that no contaminating ethanol remained. Ribonuclease-free water was added (10-20 µl, depending on the size of the RNA pellet) and the RNA concentration was measured using a nanodrop spectrophotometer (ND-1000).

2 µg RNA of each sample was reverse transcribed using random 6-mer primer (Invitrogen, Carlsbad, CA) and M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA). Samples were then analyzed in triplicate by PCR using oligonucleotides specific for ICP0 transcripts originating either from the native ICP0 start site (ICP0 TATA FW) or the CMV immediate-early minimal promoter start site (CMV TATA FW) and ICP0 exon II in reverse orientation (ICP0E2rv). The ICP4 transcripts were analyzed by PCR using oligonucleotide pair I4sen and I4ant. The sequences of oligonucleotides used

are listed in section 3.6. The PCR parameters used were: 21 cycles of 95°C for 30 sec, 58°C for 30 sec, and 72°C for 1 minute.

4.1.7. Luciferase assay

After each treatment cells were washed with PBS and dry plates were frozen at -80°C until further use. To determine luciferase activity, cells were lysed and treated following the guidelines of the kit (Luciferase assay system, E1501, Promega), and analyzed in an AutoLumat LB953 luminometer (Berthold Technologies). All experiments were performed in triplicate. Normalized luciferase activity represented in the graphs was the result of average relative light units (RLU) values obtained for each sample divided by the protein concentration.

4.1.8. Western blotting

4.1.8.1. Preparation of SDS-PAGE gels

Gels for sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE) were cast by hand using components of the Mini-PROTEAN apparatus (Bio-Rad Laboratories, Inc.). Each gel consists of a lower resolving portion and an upper stacking portion. The resolving gel was prepared by mixing the following ingredients as shown in **Table 1.**

Table 1. Composition of resolving gel

Ingredients	Volume (8% Acrylamide concentration)
Water	5.3 ml
30% Acrylamide/ Bis Acrylamide	2 ml
1.5 M Tris-Cl (pH 8.0)	2.5 ml
10% SDS	100 µl
Ammonium persulphate	100 µl
TEMED	6 µl

The ingredients were mixed in a conical centrifuge tube (15 ml) and poured smoothly in between two glass plates, one short plate and one taller plate with gel spacer of 1.5 mm, aligned and secured within a casting frame supported on a gasket of a casting stand. The solution was overlaid with double-distilled water and left to polymerize for 30 minutes.

Meanwhile, the mixture for stacking gel was prepared as follows:

Table 2. Composition of stacking gel

Ingredients	Volume
Water	4.1 ml
30% Acrylamide/Bis Acrylamide	1 ml
1 M Tris-Cl (pH 6.8)	750 µl
10% SDS	60 µl
Ammonium persulphate	60 µl
TEMED	6 µl

Once the resolving gel solidified, water was removed and the stacking gel mixture was poured over the resolving gel between the two glass plates. A 10 well or 15 well comb of 1.5 mm thickness was inserted in the stacking portion and left to polymerize for 30 minutes.

4.1.8.2. Protein sample preparation

Cells were collected, washed and resuspended in lysis buffer (50 mM Tris-HCl pH-8.0, 5 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, 10 mM sodium fluoride and 1X proteinase-inhibitor mixture (Roche)). The cell debris was removed by centrifugation (10,000 x*g* for 10 minutes) and supernatant was collected. Protein concentration of the extracts was measured using the Dc protein assay kit (Bio-Rad, Hercules, CA).

SDS sample buffer (5X) was prepared by mixing 0.25% bromophenol blue, 0.5 M dithiothreitol (DTT), 50% glycerol, 10% SDS and 0.25 M Tris-Cl (pH 6.8). 20% β- mercaptoethanol was added to the sample buffer before use. 25 µg of each cell lysates (see above paragraph) were mixed with sample buffer (0.2 volume) and heated at 100°C for 5 minutes before loading into the gel.

4.1.8.3. Gel electrophoresis

Once the stacking gel solidified, the comb was removed gently and the glass plates released from the casting frame and placed within the electrophoresis module assembly. The assembly was taken in a tank that was then filled with the electrophoresis buffer (section 3.8). Protein samples, prepared in SDS sample buffer (see above paragraph), along with a molecular weight standard (GE Healthcare) were loaded in the wells. The tank was covered with a lid bearing electrical cables to connect with a power supply with the proper polarity. Electrophoresis was carried out initially at 30

mA until the samples entered the resolving portion and then at 60 mA until the dye front reached the bottom of the gel. Once the gel run was over, the plates were removed and separated gently to take out the gel before setting transfer.

4.1.8.4. Transfer of proteins to membrane

The proteins resolved by gel electrophoresis were transferred to a piece of nitrocellulose membrane of 0.45 μm pore size (Whatman Protran) as follows:

(1) Membranes were cut into 8.5 x 5.5 cm size and equilibrated in transfer buffer for 5-10 minutes. Two pairs of Whatman filter paper, cut to similar size, and one pair of fiber pads were similarly equilibrated.

(2) The transfer was set up using the Mini Trans Blot apparatus (Bio-Rad Laboratories, Inc.). A gel sandwich was prepared within a gel holder cassette by placing sequentially one fiber pad, one pair of filter paper, the gel, the membrane, one pair of filter paper and the other fiber pad. Care was taken to eliminate any air bubble between the layers of the sandwich.

(3) The cassette was carefully closed and placed in the electrode module with the gel facing the cathode end. The module was taken in a tank that was then filled with transfer buffer (section 3.8). The tank was covered with a lid bearing cables to connect with a power supply. Transfer was carried out at 200 mA for 2 hours at 4°C, following which the blotting sandwich was disassembled and the membrane was removed. To visualize the transferred proteins, the membrane was soaked in Ponceau S dye (0.1% (w/v) Ponceau S in 5% acetic acid), after which it was washed and processed for immunodetection.

4.1.8.5. Immunodetection of proteins

(1) The membrane was blocked for 1 hour at room temperature with blocking buffer (5% skimmed milk in TBST).

(2) The membrane was incubated with primary antibody diluted in blocking buffer overnight at 4°C.

(3) The membrane was washed 3 times for 10 minutes each with TBST.

(4) The membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Sigma) diluted (1:2000) in blocking buffer for 1 hour at room temperature.

(5) The membrane was washed 3 times for 10 minutes each with TBST.

(6) Finally, the membrane was treated with Enhanced Chemiluminescence (ECL) detection reagents (PerkinElmer, Boston, MA) according to manufacturer's protocol and exposed in a GE logic 1500 imaging system (Eastman Kodak, Rochester, NY) to visualize antibody specific bands. Protein band densities were quantified using ImageJ software (Schneider et al, 2012).

4.1.9. Immunofluorescence (IF) assays

Vero cells were grown on 12 mm glass coverslips and infected with viruses at an MOI of 1. Cells were fixed at 4, 8 and 24 hours post infection by incubating in a mixture of 95% ethanol and 5% glacial acetic acid for 5 minutes at -20°C (Everett & Murray, 2005). After fixation cells were blocked in blocking buffer (PBS containing 1% FBS and 0.2% Triton X-100) for 30 minutes at room temperature. The cells were incubated with rabbit polyclonal anti-PML (1:50) and mouse monoclonal anti-ICP0 (1:50) antibodies for overnight at 4°C. After incubation cells were washed 3 times with PBS containing 1% FBS followed by incubation with secondary antibody for 1 hour at room temperature. The secondary antibodies used were Alexa-Fluor 488 conjugated anti-mouse and Alexa-Fluor 555 conjugated anti-rabbit (1:500, Molecular Probes/Life Technologies, Eugene, OR). Cell nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) (1 ng/µl, Calbiochem, Nottingham, UK) for 10 minutes at room temperature, and mounted with Fluoromount-G (Southern Biotech Birmingham, AL). The samples were examined and images were taken using an Olympus IX81 microscope. ICP0 expression in virus-infected cells was quantified using ImageJ software (Schneider et al, 2012)

4.1.10. Detection of ICP0 expression in single cell

Vero cells were grown on 12 mm glass coverslips and infected with viruses at an MOI of 0.007. Cells were fixed at 24 hours post infection and stained with anti-ICP0 antibody as described in the section 4.1.9. The secondary antibody used was Alexa-Fluor 488 conjugated anti-mouse (1:500). Mock-infected cells were used as negative control for ICP0 staining. Cell nuclei were counterstained with DAPI for 10 minutes at room temperature and mounted with Fluoromount-G. Cells on coverslips were photographed using an Olympus IX81 microscope. Images for all the samples were captured during the same session at exposure times of 47 and 1 ms for green and blue fluorescence respectively. The pixel density of ICP0 expression in single cells infected by viral samples was quantified using ImageJ software (Schneider et al, 2012). The background for staining was normalized by subtracting the mean pixel density of the mock-infected cell from the mean pixel density value of each sample.

4.1.11. Detection of β-galactosidase expression

The culture medium was removed from the infected plate and cells were washed twice with 2 ml PBS and fixed with 1ml 4% paraformaldehyde (PFA) for 10 minutes at room temperature. The cells were washed three times with PBS and incubated at 37°C with 2 ml of pre-warmed X-gal solution (PBS supplemented with 2 mM $MgCl_2$, 5 mM potassium ferrocyanide $[K_4Fe(CN)_6].3H_2O$, 5 mM potassium ferricyanide $K_3Fe(CN)_6$ and 1 mg/ml X-gal [5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside dissolved in Dimethylformamide]) until stained. The X-gal stain was then removed and the cells were washed with PBS.

4.1.12. Bacterial cell culture

All bacterial cultures were grown in LB (lysogeny broth; (Bertani, 1951)) or semi-solid agar plates containing LB and bacterial agar (1.5% w/v). LB was autoclaved at 121°C for 20 minutes at 15lb/inch^2. Antibiotics were added as required just before use.

4.1.13. Chemically competent cells preparation

Competent cells used for transformation of plasmid DNA were prepared using the standard $CaCl_2$ technique. A single bacterial colony was grown overnight in 3 ml of LB containing no antibiotics at 37°C. The saturated overnight culture was diluted 500-fold in 100 ml of LB containing no antibiotics. The cells were allowed to grow at 37°C until the optical density (OD) derived from absorbance measured at 600 nm reached between 0.3-0.4. Cells were pelleted by centrifugation at 10,000 x*g* for 10 minutes at 4°C. The supernatant was discarded and the pellet resuspended gently in 25 ml of ice-cold 100 mM $MgCl_2$. Cells were pelleted as before, resuspended in 4 ml of ice-cold 100 mM calcium chloride ($CaCl_2$) and kept on ice for 10 minutes. Glycerol was added to the bacterial suspension to reach the final concentration of 10% (v/v). Competent cells were aliquoted and stored at -80°C until further use.

4.1.14. Bacterial transformation

Bacterial chemically competent cells were thawed on ice and 50 µl of cells were pipetted into 1.5 ml microcentrifuge tubes for each transformation. 1-5 µl of ligation mix or 50 ng of plasmid DNA weas added to the cells, mixed gently and incubated on ice for 30 minutes. The cells were given heat shock for 30 sec in a 42°C water bath and tubes were placed on ice for 10 minutes. 950 µl of pre-warmed SOC (super optimal broth (Hanahan, 1983) with 20 mM added glucose) was added to each tube and incubated at 37°C for 1 hour with agitation (1100 rpm). The transformed cells were plated in two different volumes on LB agar plates containing the appropriate antibiotic and incubated overnight at 37°C in a standard bacterial incubator. Two different volumes were taken to ensure that at least one plate will have well-spaced colonies.

4.1.15. Colony screening by PCR

Multiple bacterial colonies for each insert were picked up and amplified by PCR using one oligonucleotide annealing to the vector and another to the insert. PCR products were resolved on an agarose gel and positive clones were detected by the presence of band at the predicted size.

4.1.16. Plasmid amplification

Bacterial cultures were grown for the positive clones and plasmid DNA was extracted using a Qiagen Plasmid Mini/ Maxi preparation kit following the guidelines provided.

4.1.17. DNA sequencing

Plasmid (100 ng/µl) and BAC (200 ng/µl) DNA were sequenced by the Secugen S.L sequencing service (Madrid, Spain). Oligonucleotides were synthesized by Isogen Life Science (Veldzigt, the Netherlands) and were used at a concentration of 5 µM.

4.2. CELL CULTURE TECHNIQUES

All cell culture solutions, media and supplements were obtained from Gibco. All manipulations of cells were carried out under sterile conditions using standard aseptic techniques.

4.2.1. Freezing and recovery of cells

Cells from one 100 mm cell culture dish were trypsinized, resuspended in 1.8 ml of freezing mixture (10% (v/v) dimethyl sulfoxide (DMSO) and 90% (v/v) FBS) and aliquoted in 2 cryovials, which were slowly cooled to -80°C by keeping in a cryobox containing 2- propanol and then stored in liquid nitrogen tank.

DMSO is toxic above 4°C and thus when recovered, cells were rapidly thawed and centrifuged to minimize the toxic effects of the cryoprotectant. Cell pellets were gently resuspended in 1 ml pre-warmed F10 medium and slowly transferred to 100 mm cell culture dishes with F10 medium.

4.2.2. Cell passage

Cells were grown in 100 mm cell culture dish in appropriate media (section 3.3). They were incubated at 37°C/5% CO_2 humidified cell incubator and routinely passaged when 75-80% confluent. The growth medium was carefully removed and the cell monolayer was gently washed with sterile PBS and incubated with 2 ml Tryp-LE (for 100 mm cell culture plate) until the cells were detached from the surface of the plate. 2 ml of F10 medium were added to the plate to neutralize the effect of Tryp-LE. The trypsinized cells were centrifuged in a 15 ml conical centrifuge tube at 200 x*g* for 5 minutes. The obtained cell pellet was resuspended in a small volume of fresh medium and seeded at a ratio of 1:10 in appropriate cell culture medium (section 3.3).

4.2.3. Cell line transfection

Cells were transfected using the Lipofectamine-LTX and Plus reagent. A day prior to transfection, 1.6 X 10^5 cells were seeded in a 35 mm cell culture dish to achieve 70% cell confluency on the day of transfection. In a sterile 1.5 ml microcentrifuge tube, 1 µg (plasmid) - 2 µg (BAC) DNA was diluted into 100 µl of Opti MEM medium and mixed with Plus reagent in a ratio of 1:2 (plasmid DNA) or 1:3 (BAC DNA). The mixture was incubated for 5 minutes at room temperature. In another microcentrifuge tube, 12 µl of Lipofectamine-LTX reagent was diluted in 100 µl of Opti-MEM and added to the DNA-Plus mixture. The Lipofectamine, DNA and Plus mixture was allowed to form

complexes over 20 minutes at room temperature. The cell culture medium was replaced with 0.8 ml Opti-MEM and the Lipofectamine-DNA-Plus mixture (200 µl) was gently added to the cells in drops. After 4 hours, the medium with transfection mixture was replaced with pre-warmed cell culture medium and cells were grown under normal conditions.

4.2.4. Construction of stable cell lines

4.2.4.1. Vero cell line expressing the reverse tetracycline-responsive transactivator (rtTA)

Cell lines expressing rtTA (Vero-rtTA) were generated by transfection of Vero cells with the plasmid pLD-puro-2A-rtTA-TcVA (Mak et al, 2010) using Lipofectamine-LTX and Plus reagent as described in the section 4.2.3. Stably transfected clones were selected and maintained in F10 medium supplemented with puromycin (3 µg/ml). After recloning twice, the cell line with the highest inducibility of the rtTA transgene was identified by luciferase assay (E1501, Promega, Madison, WI) after transfection with a tetO-containing reporter plasmid (Gossen & Bujard, 1992).

4.2.4.2. Vero cell line expressing the tetracycline repressor- Krüppel associated box (KRAB) (tetR-KRAB) fusion protein:

Two oligonucleotides with complementary base pairing were synthesized; XLNK (TCGAGATCTACTAGT) and NLNK (CATGACTAGTAGATC). Both oligonucleotides were annealed together by mixing in equal volumes (at equimolar concentration) in a 1.5 ml microcentrifuge tube. The tube was heated in a standard heat block at 90-95°C for 3-5 minutes and then allowed to cool slowly at room temperature. This annealing generated a cohesive end compatible with those generated by *Xho*I restriction enzyme at one end of the annealed fragment (XLNK-NLNK). The *Eco*RI/*Nco*I tetR-KRAB fragment generated from double digestion of the plasmid pLVPRT-tTRKRAB (Szulc et al, 2006) was ligated to the *Xho*I-XLNK-NLNK fragment. This ligation destroys the *Nco*I site from the tetR-KRAB fragment. The fragment obtained from the ligation (*Xho*I/*Eco*RI KRAB-XLNK-NLNK) was purified by ethanol precipitation method and cloned into the pEF-IRESP plasmid (Hobbs et al, 1998) digested with *Xho*I and *Eco*RI to generate the plasmid pEFKRAB.

Cell lines expressing tetR-KRAB (Vero-tetRKRAB) were generated by transfection of Vero cells with the plasmid pEFKRAB using Lipofectamine-LTX and Plus reagent as described earlier (see section 4.2.3). Stably transfected clones were selected and maintained in F10 medium supplemented with puromycin (3 µg/ml). After recloning twice, the cell line with the highest tetR-KRAB-mediated transgene repression was identified by luciferase assay after transfection with a tetO-containing reporter plasmid (Gossen & Bujard, 1992).

Both cell lines Vero-rtTA and Vero-tetRKRAB were used in the present study to analyze tetO-containing viral mutants.

4.3. HSV-1-BAC RECOMBINEERING

4.3.1. Generation of HSV-1 mutants by homologous recombination

The parental virus strain used in this study was strain F of HSV-1(Ejercito et al, 1968). The viral genome cloned in a bacterial artificial chromosome (BAC) plasmid (Tanaka et al, 2003) was used for the construction of all the mutants by lambda Red-mediated homologous recombination ((Muyrers et al, 1999; Zhang et al, 1998) reviewed in (Muyrers et al, 2001)) following the guidelines provided with a commercial kit (Gene bridges, K001, version 2.7). Briefly, it involved the following steps:

4.3.1.1. Generation of the linear targeting fragment flanked by homology arms for construction of HSV-1 mutants

A targeting fragment containing an antibiotic selection marker gene flanked by homologous sequence arms stretches of DNA shared by the two molecules that recombine, was generated by PCR using customized oligonucleotides. The 5' oligonucleotide consisted of a 50 nucleotide homology arm (which allows recombination with the homologous sequence 5' of the intended insertion site), followed by a 20-nucleotide stretch which primes the 5' end of the PCR amplification of the selection marker. The 3' oligonucleotide was similarly constructed, with a stretch of 50 nucleotides that is homologous to the region 3' of the intended insertion site, followed by a 20-nucleotide stretch that primes the 3' end of the PCR amplification of the selection marker.

For ΔIR construction, the targeting fragment was generated by PCR amplification of the beta lactamase (*bla*) gene (conferring ampicillin resistance) using oligonucleotides UL5A and US3A (see section 3.6) and plasmid pSK+KanaRpsL as template DNA. The recombination targeting fragments for mutants TetΔ34.5TG, Tet-in and TetΔ34.5 were respectively generated by digestion of plasmids pΔ34.5TGT0, pTGT0 and pΔ34.5T0 with *Eco*RI and *Pvu*I restriction enzymes. Plasmid pΔ34.5TGT0 was constructed by ligating the *Xba*I/*Xho*I RP-Kan^R cassette into ptetICP0B. The RP-Kan^R cassette is composed of the *E. coli* ribosomal S12 (*rpsL*) gene (RP) which results in streptomycin sensitivity and the aminoglycoside phosphotransferase (*aph*) gene which confers kanamycin resistance (Kan^R). The RP-Kan^R was generated by PCR using oligonucleotides ATN5 and KANA3P and pSK+KanaRpsL as template DNA (see section 3.6). Plasmid pTGT0 was derived in a similar manner as pΔ34.5TGT0 but using oligonucleotides TGRAT and KANA3P. The sequences of the oligonucleotides used for PCR are listed in section 3.6. Plasmid pΔ34.5T0 was generated by triple ligation of the 1.4 kb *Sac*I/*Bam*HI fragment from pAXKS, the 1.76 kb *Xba*I/*Sac*I fragment from pΔ34.5TGT0 and the 2.6 kb *Xba*I/*Bam*HI fragment from pΔ34.5TGT0.

For mutant TetΔ34.5TG-K, linear fragment was generated by digestion of the plasmid pI0prtTRZ with *Pvu*II restriction enzyme.

For mutant TetΔTG construction, the targeting fragment was generated by PCR amplification of the RP-Kan^R cassette using oligonucleotides ΔTGR5P and TetΔTGK3P (see section 3.6) and pSK+KanaRpsL as template DNA.

4.3.1.2. Transformation with Red/ET expression plasmid

Glycerol stock of *E. coli* strain DH10B (Durfee et al, 2008; Grant et al, 1990) containing the target HSV-1 BAC was streaked on a LB agar plate conditioned with chloramphenicol (15 μg/ml). The plate was incubated overnight at 37°C in a bacterial incubator.

Day 1: 1 colony from the streaked plate was picked and inoculated in a 1.5 ml microcentrifuge tube containing 1 ml of LB conditioned with chloramphenicol (15 μg/ml). A small hole in the lid was made for air passage. The tube was incubated at 37°C overnight with agitation (1100 rpm).

Day 2: 2 microcentrifuge tubes were set up with 1.4 ml of LB conditioned with chloramphenicol (15 μg /ml) and inoculated with 30 μl of the overnight culture. Tubes were then incubated at 37°C for 2 hours with agitation (1100 rpm).

After incubation, electrocompetent cells were prepared by centrifuging bacterial culture at 11,000 x*g* for 1 minute at 2°C. The pellet was resuspended in ice-cold water and centrifuged again (11,000 x*g*, 2°C, 1 minute). This was repeated twice more and the cell pellet was resuspended in 50 μl ice-cold double-distilled water. Care was taken to maintain 4°C throughout the process.

For transformation, 1 μl of the pRed/ET plasmid (100 ng) was added to the electrocompetent cells of one of the microcentrifuge tube and mixed gently. The other tube was used as electroporation control (without pRed/ET). Electroporation was performed in ice-cold cuvettes (Bio-Rad Laboratories, Inc., 1 mm electrode gap) using a Bio-Rad MicroPulser at 1.8 kV, untruncated time setting. After electroporation, 1 ml of pre-warmed SOC (super optimal broth (Hanahan, 1983) with 20 mM added glucose) medium was added to the cuvettes to facilitate recovery of the cells which were then incubated at 30°C for 1 hour with shaking. Cells were spread onto LB agar plates containing chloramphenicol (15 μg/ml) and tetracycline (3 μg/ml) and incubated overnight in bacterial incubator at 30°C.

4.3.1.3. Insertion in the HSV-1 genome of linear fragments with desired mutations using selection markers

Day 1: A single colony was picked from LB plate containing Red/ET plasmid and target HSV-1 BAC and inoculated into a 1.5 ml microcentrifuge tube containing 1 ml LB broth with antibiotics (chloramphenicol 15 μg/ml, tetracycline 3 μg/ml). The tubes were incubated overnight at 30°C with agitation (1100 rpm).

Day 2: Four 1.5 ml microcentrifuge tubes (2 for experiment and 2 for control) were set up with 1.4 ml fresh LB supplemented with 15 μg/ml chloramphenicol and 3 μg/ml tetracycline. The tubes were

inoculated with 30 µl of the saturated overnight culture of DH10B containing the target BAC and pRed/ET. All the four tubes were incubated at 30°C until the OD derived from absorbance measured at 600 nm reached between 0.3-0.4. Expression from the pRed/ET plasmid was induced in two tubes (1 from the experiment and 1 from the control) by addition of 50 µl of 10% L-arabinose and incubation for 45 minutes at 37°C before chilling on ice for 15 minutes. The electrocompetent cells were prepared as described above (see section 4.3.1.2). For transformation, 100 ng of the linear targeting fragment was added to the electrocompetent cells on ice (both L-arabinose-induced and uninduced experimental tubes). Electroporation was performed on all the four cultures (induced and uninduced experimental tubes, induced and un-induced control tubes) in ice-cold cuvettes (1 mm electrode gap) using a Bio-Rad MicroPulser at 1.8 kV, untruncated time setting. After electroporation, 1 ml of pre-warmed SOC medium was added to the cuvettes to facilitate recovery of the cells which were then incubated at 37°C for 1 hour with shaking, inoculated onto LB agar plates containing 15 µg/ml chloramphenicol and either 50 µg/ml ampicillin (for ΔIR) or 15 µg/ml kanamycin (for TetICP0 mutants) or 25 µg/ml zeocin (for TetΔ34.5TG-K) and incubated overnight at 37°C (restrictive temperature for pRed/ET). The next day, several independent colonies were picked and tested for correct recombination by colony PCR or restriction digest analysis of purified BAC DNA.

4.3.1.4. Generation of the HSV-1 mutant Tet-inR by RP-kanamycin counter selection

The Tet-inR mutant was generated by negative selection to remove the RP-Kan^R dual marker which confers streptomycin sensitivity (Wang et al, 2009). The 100-base pair single-stranded oligonucleotide (C3DRK; see section 3.6) consisting of the 50 nucleotides flanking either side of the RP-Kan^R cassette was used as the targeting fragment to generate the deletion in the Tet-in mutant by lambda red-mediated homologous recombination as described above, and the Tet-inR mutant was selected by growth in medium containing 5 mg/ml streptomycin.

4.3.2. Cre recombinase-mediated site-specific DNA insertion into HSV-1 BAC in *E. coli*

Mutant TetΔ34.5TG-O was generated by Cre recombinase-mediated site-specific recombination (Kim et al, 1998). Electrocompetent bacterial cells containing HSV-1 BAC DNA (TetΔ34.5TG) were prepared as described above in the section 4.3.1.2. The cells were electroporated with a mixture containing 10 ng each of the temperature sensitive (ts) helper plasmid pCTP-T (which expresses Cre recombinase *in trans*) and the insertional shuttle plasmid pLOsG_Z (which contains a *loxP* site, oriS, the green fluorescent protein (GFP) reporter gene (*gfp)* and the *Sh ble* gene (conferring zeocin resistance)). After electroporation, cells were resuspended into 1 ml SOC medium containing heat-inactivated tetracycline (20 µg/ml) to induce Cre expression and incubated for 4 hours at 30°C (permissive temperature for the helper plasmid pCTP-T) with shaking. The integrated recombinants

(TetΔ34.5TG-O) were selected at 43°C (restrictive temperature for the pCTP-T) on LB agar plates supplemented with chloramphenicol (15 μg/ml), kanamycin (15 μg/ml) and zeocin (25 μg/ml).

4.3.3. Verification of modified HSV-1 BAC by DNA sequencing

DNA of HSV-1 mutants were sent to the sequencing service for the final confirmation using oligonucleotides reading across the recombination junction. The oligonucleotides used for sequencing of HSV-1 mutants are listed in **Table 3.**

Table 3. Oligonucleotides used for sequencing of HSV-1 mutants.

Mutant	Oligonucleotides Used for Sequencing
ΔIR	AMP5'OUT, AMP3'OUT
TetΔ34.5TG	RPREV, SEQK5
Tet-in	RPREV, TN5PROM, SEQK5
Tet-inR	C3DRK1, C3DRK2
TetΔ34.5	RPREV, SEQK5, C6B
TetΔTG	SEQK5, TN5PROM, I34.53OUT
TetΔ34.5TG-K	KRAB3P, KRAB200, MARIZEO
TetΔ34.5TG-O	SU1LOXHSV, SU2LOXHSVUL4

4.4. VIRAL PROPAGATION TECHNIQUES

4.4.1. Viral stock preparation

4.4.1.1. Seed stock

Approximately 1.6 X 10^5 Vero cells were seeded in a 35 mm cell culture dish to obtain 70% cell confluency on the day of transfection. Cells were transfected with 2 μg of BAC DNA encoding viral genome using Lipofectamine-LTX and Plus reagent as described earlier in the Methods. After 4 hours of transfection, cells were overlaid with 0.7% sterile agarose dissolved in F2 medium (DMEM supplemented with 2% FBS, 100U/ml penicillin and 100 μg/ml of streptomycin) and incubated at 37°C until the viral plaques were visible. A single plaque was picked using a P 20 Gilson pipette, stabbing through the agarose into the cells. The agarose plug plus the cells was transferred into a 0.5

ml microcentrifuge tube containing 40 µl of FBS and stored in -80°C until further use. A single plaque was used to infect fresh Vero cells seeded in a 35 mm culture dish. Cells were harvested when cytopathic effects (CPE, generally 1-2 days) became visible in all cells, by three freeze-thaw cycles followed by sonication to release intracellular viral particles into the supernatant. Cell debris was removed by centrifugation (2,000 x*g* for 5 minutes) and clarified supernatant was collected and stored at -80°C in aliquots.

4.4.1.2. Master stock

The viral seed stock was then amplified to prepare master stock. Approximately 2 X 10^6 Vero cells were seeded in a 100 mm cell culture dish and infected with 50 µl of seed stock in 6 ml of F2 medium. After 1-2 days when cells became round but remained anchored to the culture dish, they were scraped into their own medium using a sterile plastic cell scraper. The viral preparation was stored in aliquots at -80°C after completing 3 cycles of freeze-thaw and sonication and centrifugation steps as described for seed stock preparation.

4.4.2. Viral stock titration

Virus titers were measured by infecting Vero cells with different dilutions of viral preparation in F2 medium. After absorption for 90 minutes, the medium was replaced with 0.7% sterile agarose dissolved in F2 medium. After overnight incubation, cells were fixed with 4% PFA and the agarose was removed. Cells were incubated in blocking buffer (PBS containing 1% FBS and 0.2% Triton X-100) for 30 minutes at room temperature and subsequently incubated with a rabbit polyclonal anti-HSV-1 antibody (1:1000) for 2 hours at room temperature. After incubation, cells were washed 3 times in PBS and then incubated with anti-rabbit secondary antibody/peroxidase conjugate (1:2000, Sigma-Aldrich, St. Louis, MO) for 1 hour at room temperature. Peroxidase-labeled cells were visualized by incubating in a mixture of 0.05% 3, 3'-Diaminobenzidine (DAB; Acros Organics) and 0.015% hydrogen peroxide (H_2O_2; Panreac, Barcelona, Spain) for 5 minutes. The DAB mixture was removed and cells were washed once with PBS to stop the staining process. Bright field photographic images were used to count the number of HSV-1 positive cells (stained brown) using the ImageJ cell counter plug-in (Schneider et al, 2012) to calculate infectious HSV-1 particle titer.

4.4.3. *In vitro* viral growth curve

For viral growth curve experiments, approximately1.8 X 10^5 cells were seeded in 35 mm cell culture dishes and infected with viruses at stated MOIs. Cells were incubated at 37°C for 90 minutes to allow the virus to be absorbed after which inocula were replaced with F2 medium and the cultures incubated at 37°C for indicated times. Progeny viruses were harvested by lifting the cells with a plastic scraper into the medium which was then subjected to three freeze-thaw cycles followed by sonication. The cell lysate was centrifuged at 2,000 x*g* for 5 minutes and titration of the viral supernatant was performed on monolayer of Vero cells as described in the above paragraph.

For transfection experiments, 2 μg of BAC DNA encoding viral genome was transfected in a 35 mm cell culture dish of Vero cells using Lipofectamine-LTX and Plus reagent. Cells were harvested at specified times and viral titers were measured over fresh Vero cells.

4.4.4. One step growth kinetics

For single cycle growth assays Vero cells were infected at an MOI of 5 for 1 hour, after which the cells were washed twice with PBS and re-incubated in F2 medium until harvested for virus titration. Supernatants and cells were harvested separately to estimate titer of extracellular and intracellular viral particles.

4.4.5. Focus formation assay

Vero cells were infected at the indicated MOI for 90 minutes, after which the cells were overlaid with 0.7% sterile agarose dissolved in F2 medium for stated time points. Cells were then fixed with 4% PFA and stained with rabbit polyclonal anti-HSV-1 antibody as described in the virus titration section (4.4.2). Cells were imaged by bright field microscopy and focus area was measured using ImageJ software (Schneider et al, 2012).

4.4.6. Drug treatments

To inhibit protein synthesis, 200 μM cycloheximide (Sigma-Aldrich, St. Louis, MO) was added to Vero cells 0.5 hour prior to viral inoculation and was maintained in cultures until the viral inoculum was replaced with fresh F2 medium containing either 300 μM acyclovir (Calbiochem, Billerica, MA) or 10 μg/ml actinomycin D (Sigma-Aldrich, St. Louis, MO) or no drug (vehicle only).

4.5. STATISTICAL ANALYSIS

Statistical comparison of the data sets was performed by Student's *t* test. The differences are given with their corresponding *P* value, which is the probability that the difference occurred merely by chance under the null hypothesis.

Results

5. RESULTS

5.1. Generation of the ΔIR mutant: deletion of the internal repeat (IR) sequences has little effect on HSV-1 growth in Vero cells

The WT HSV-1 genome consists of two repeated regions- IR and TR. The ΔIR mutant was constructed by deleting the IR sequences from the WT genome using bacterial homologous recombination as described in the Methods. The IR deletion removes one copy of all the duplicated regions present in the viral genome which include the genes encoding ICP0, ICP4, ICP34.5, the packaging signal and oriS. This deletion ensures unambiguous targeting of any subsequent mutagenesis and increases viral genomic stability by eliminating rearrangements due to isomerization of the unique segments (U_L/U_S). The structure of the ΔIR genome compared to the WT genome is illustrated in **Figure 9A**. The HSV-1 strain F (Ejercito et al, 1968) genome cloned in a bacterial artificial chromosome (BAC) (Tanaka et al, 2003) has four *Bam*HI restriction enzyme sites in the IR region. Candidate ΔIR mutants generated by homologous recombination were checked for loss of these restriction sites by digesting purified BAC DNA with *Bam*HI enzyme followed by electrophoresis in a 0.8% agarose gel (**Figure 9B**). The deletion boundaries were further confirmed by PCR and DNA sequencing. The ΔIR master stock was generated as described in Methods and could be grown up to titers similar to WT.

The effect of IR deletion on HSV-1 replication was first tested by evaluating multi-cycle growth kinetics in Vero cells using an MOI of 0.1. The growth curve exhibited by the ΔIR mutant is indistinguishable from that of WT, indicating a negligible effect of the IR deletion on overall viral titer (**Figure 9C**). Measurement of viral progeny release in single step kinetics using an MOI of 5 also revealed little differences in the rate of increase in infectious particle concentrations either in the intracellular or extracellular fractions although at early time points the titer of extracellular ΔIR particles was 30%-70% of that of WT, suggesting a slight defect in virion egress which is no longer evident at 24 hours post infection (**Figure 9D**).

Following this observation I examined the effects of IR deletion on viral replication initiated in the absence of virion components by transfection of viral genomic DNA. The WT or ΔIR BAC DNA was transfected into Vero cells and viral titer was measured in cell extracts harvested at 24, 32, 48 and 56 hours post transfection. As a control for transfection efficiency of the BAC DNAs, the number of immunopositive cells for HSV-1 antigen was counted in parallel transfected samples fixed at 8 hours post transfection. This value was used to normalize respective WT and ΔIR titers measured at all the time points. The ΔIR mutant showed a reduced viral titer per transfected cell compared to WT in this assay (**Figure 9E**), indicating a slight defect in growth in the absence of virion components.

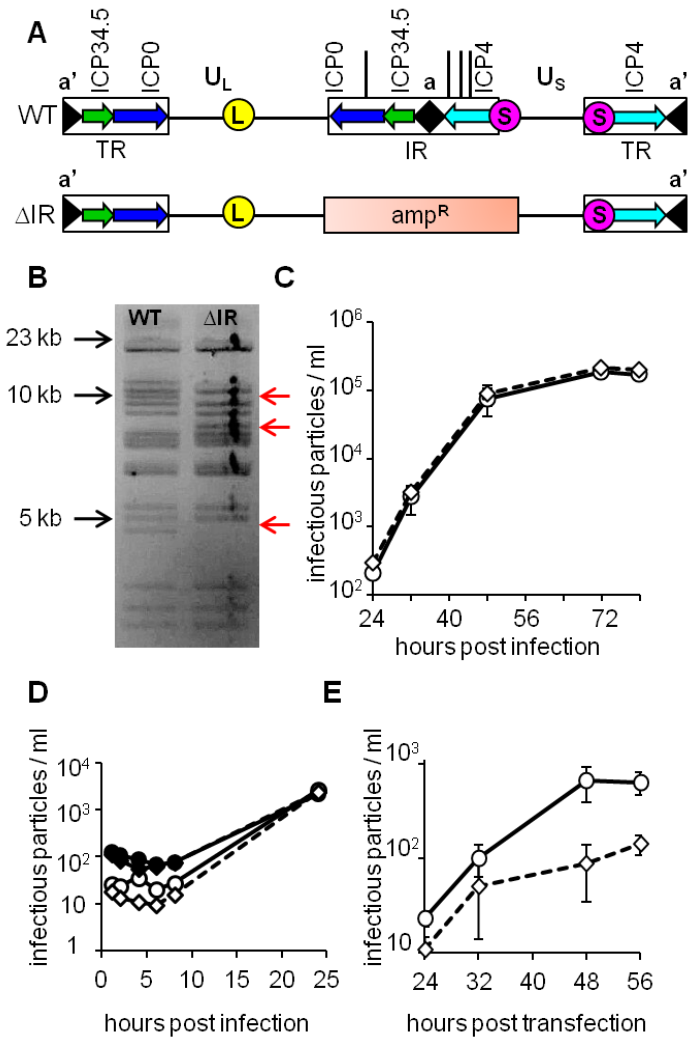

Figure 9. Characterization of the HSV-1 mutant ΔIR deleted for the internal repeat region.

A. Schematic representation of genomes of wild type HSV-1 (WT) and ΔIR. The unique long (U_L) and the unique short (U_S) segments are shown flanked by the internal repeat (IR) and the terminal repeat (TR). The unique origin of replication oriL is present in the U_L segment (**L** enclosed by circle). The immediate early genes encoding ICP0 and ICP4, the origin of replication oriS (**S** enclosed by circle) and the packaging signal (**a** and **a'**) are present in the WT genome in duplicate copies, one of which has been deleted in the ΔIR mutant. The ΔIR mutant was constructed by replacing one copy of *ICP0*, *ICP4*, *ICP34.5*, one packaging signal and one origin of replication by the beta lactamase (*bla*) gene conferring ampicillin resistance (amp^R). Vertical lines over IR represent four *Bam*HI restriction enzyme sites which are eliminated by the ΔIR mutation.

B. Agarose gel electrophoresis pattern of WT and ΔIR BAC DNA digested with *Bam*HI enzyme. Leftward red arrows show band differences due to the loss of the *Bam*HI sites in the IR region.

C. Growth kinetics of WT (circles, solid line) and the ΔIR mutant (diamonds, dotted line) in Vero cells infected at a multiplicity of infection (MOI) of 0.1.

D. Growth kinetics of WT and the ΔIR mutant in Vero cells infected at an MOI of 5 showing titers of intracellular (in) viral particles (WT;solid circles and ΔIR; solid diamonds) and extracellular (ex) viral particles (WT; open circles and ΔIR; open diamonds).

E. Growth kinetics of WT (circles, solid line) and the ΔIR mutant (diamonds, dotted line) after transfection of BAC DNA in Vero cells.

Graphs in C and E show mean values and error bars indicating the standard deviation of three independent experiments.

These results indicate that though ΔIR initially exhibits slow replication in the absence of virion proteins, the final titer after multi-cycle replication in Vero cells is not adversely affected, indicating that only a single copy of all the genetic elements in HSV-1 is sufficient for viral replication in culture.

5.2. Generation of ΔIR mutants with ICP0 promoter modifications

The ΔIR backbone was next used to generate mutants in the region upstream of the single ICP0 open reading frame to identify possible strategies to impose control over HSV-1 lytic replication. A first set of mutant genomes was constructed (**Figure 10**) in which *tet* operator (tetO) sites were inserted: (1) between the ICP0 transcription start site (TSS) and the TAATGARAT motif, displacing the latter 1.5 kb further upstream (**Figure 10A**, mutant Tet-in); (2) replacing all of the ICP0 promoter up to TAATGARAT as well as the upstream *ICP34.5* gene (**Figure 10A**, mutant TetΔ34.5TG). Deletion of both copies of the *ICP34.5* gene in the WT genome has previously been observed to be non-deleterious for viral replication in cell lines (Chou et al, 1990; Kesari et al, 1998) and so to confirm that ICP34.5 is also non-essential for viral growth in culture in the context of the ΔIR backbone, a third construct was generated in which tetO sites were inserted to replace only the *ICP34.5* gene, leaving the ICP0 promoter region intact (**Figure 10A**, mutant TetΔ34.5).

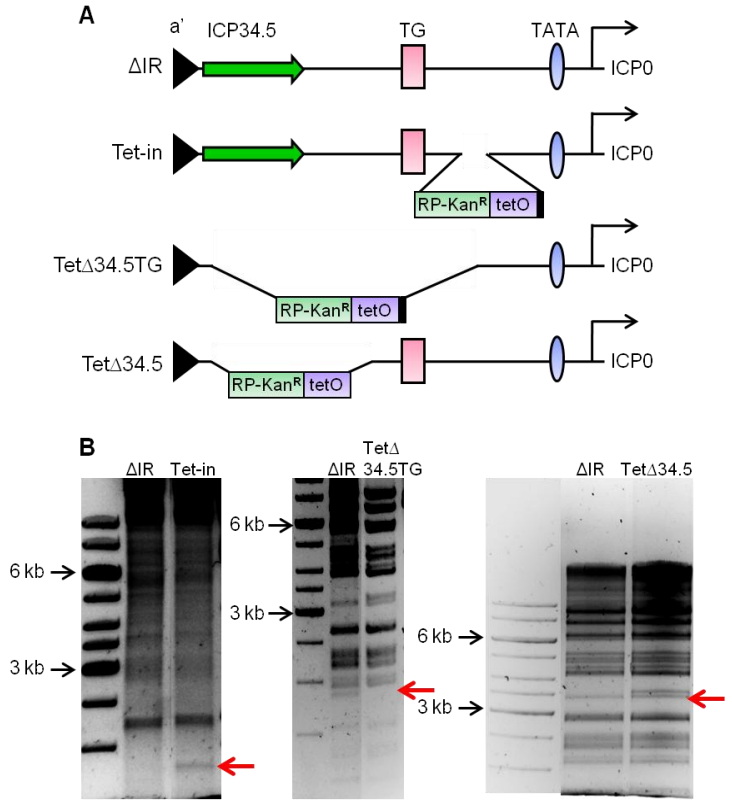

Figure 10. Mutation of the ICP0 promoter region in ΔIR by tetO insertion.

A. Diagram of the HSV-1 terminal repeat (TR) region showing the *ICP34.5* gene, the consensus TAATGARAT motif (TG) and the TATA box (TATA) upstream of the transcription start site (rightward arrow) of the *ICP0* gene, all of which are present as a single copy in the ΔIR mutant. Three ICP0 promoter mutants were constructed by insertion of the RP-Kan^R/tetO cassette, composed of: the *E. coli* ribosomal S12 (*rpsL*) gene (RP) which results in streptomycin sensitivity; the aminoglycoside phosphotransferase (*aph*) gene which results in kanamycin resistance (Kan^R); target-binding sites for the *E. coli* tetracycline repressor consisting of 7 *tet* operator (tetO) sequences. In the Tet-in mutant, the RP-Kan^R/tetO cassette was inserted in between the TG motif and the TATA box. In the TetΔ34.5TG mutant, the RP-Kan^R/tetO cassette replaced the whole region encompassing the *ICP34.5* gene up to and including the TG motif. In the TetΔ34.5 mutant, only the *ICP34.5* gene was replaced by the RP-Kan^R/tetO cassette.

B. Agarose gel electrophoresis analysis of ΔIR-derived genomes containing mutations in the ICP0 promoter treated with restriction enzymes. BAC DNA encoding the Tet-in mutant was digested with *Ase*I and that encoding the TetΔ34.5TG and TetΔ34.5 mutants was digested with *Nco*I restriction enzymes. The parental BAC encoding ΔIR was used as a control in all the digestions. Leftward red arrows show band differences due to the mutations in the *ICP34.5* gene and ICP0 promoter region.

All three TetICP0 mutants listed above were generated by homologous recombination in bacteria using linear targeting fragments derived from plasmids pTGT0, pΔ34.5TGT0 and pΔ34.5T0 respectively as described in the Methods. The structures of the mutant genomes compared with the ΔIR genome were verified by digestion of their encoding BAC DNA with restriction enzymes followed by electrophoresis in a 0.8% agarose gel: for the Tet-in mutant, *Ase*I was used, while for the mutants TetΔ34.5TG and TetΔ34.5, *Nco*I was used. Leftward red arrows in **Figure 10B** indicate changes in restriction digest patterns of BAC DNA of mutants Tet-in, TetΔ34.5TG and TetΔ34.5 compared to that of ΔIR. The deletion junctions were further confirmed by PCR and DNA sequencing.

5.3. Phenotypic characterization of TetICP0 mutants

To test whether mutations in the ICP0 promoter and its upstream region produce phenotypic changes, the TetICP0 mutants were characterized by timing the appearance of the first plaques and quantifying the spread of infection *in vitro*. BAC DNA encoding either the WT, ΔIR, Tet-in, TetΔ34.5TG or TetΔ34.5 mutant genome was transfected into Vero cells in semi-solid medium, and stained with anti-HSV-1 antibody 1, 2, 4 and 6 days post-transfection.

5.3.1. Plaque formation

The WT virus grew rapidly in Vero cells with the appearance of the first plaque early on day 3 after transfection. On day 4 all cells in the plate appeared infected with round morphology and many medium to big plaques were evident. Nearly all cells were lysed early on day 6 after transfection.

Compared to WT, the ΔIR mutant showed reduced viral replication with the appearance of the first plaque late on day 3 after transfection. This observation correlates with the earlier result (section 5.1) where ΔIR showed slow replication in absence of virion proteins. Transfection of DNA encoding the modified genomes revealed different viral growth for each of the three TetICP0 mutants. The Tet-in mutant exhibited slower viral production in Vero cells with plaques appearing 4 days after transfection, compared to 3 days for the ΔIR mutant, consistent with a negative effect of the displacement of the TAATGARAT element on ICP0 expression. On day 6, numerous Tet-in plaques were filled in by overgrowth of surrounding non-productive cells. In TetΔ34.5TG-transfected cells, syncytia and very small plaques were often visible on day 4 but all such signs of viral growth disappeared by day 6. In cells transfected with mutant TetΔ34.5 the first plaque appeared late on day 3, similar to ΔIR and the number of plaques increased from day 3 to day 6.

5.3.2. Infection spread

In the present study, infection spread was quantified by measuring the sum of the area of foci at various time points. Small infection foci of WT were observed on day 1 post-transfection which spread exponentially to cover the whole plate by day 2 (**Figure 11A**). On day 4 numerous large plaques were evident in the cell monolayer which was lysed completely on day 6 due to the viral

replication. In the ΔIR-transfected plate, small foci were observed on day 1 which increased on average, 225-fold in area by day 6 resulting in infection spread to the whole plate (**Figure 11A** and **11B**). Compared to WT, ΔIR replication appeared slower, since on day 6, cells were observed infected with visible plaques but remained attached to the plate (**Figure 11A**). The infection spread in TetICP0 mutants- Tet-in and TetΔ34.5 appeared slower compared to the parental ΔIR mutant (**Figure 11**, approximately 21% and 30% of ΔIR infection spread on day 6) The TetΔ34.5TG mutant containing a deletion in *ICP34.5* gene and a compromised ICP0 promoter was observed to have the slowest and most restricted infection spread amongst all the constructed TetICP0 mutants (approximately 5% of ΔIR infection spread on day 6).

Taken together, these results demonstrate that although ICP34.5 is nonessential for viral replication (Chou et al, 1990); its role becomes significant when ICP0 expression is compromised, such as in the case of the TetΔ34.5TG mutant.

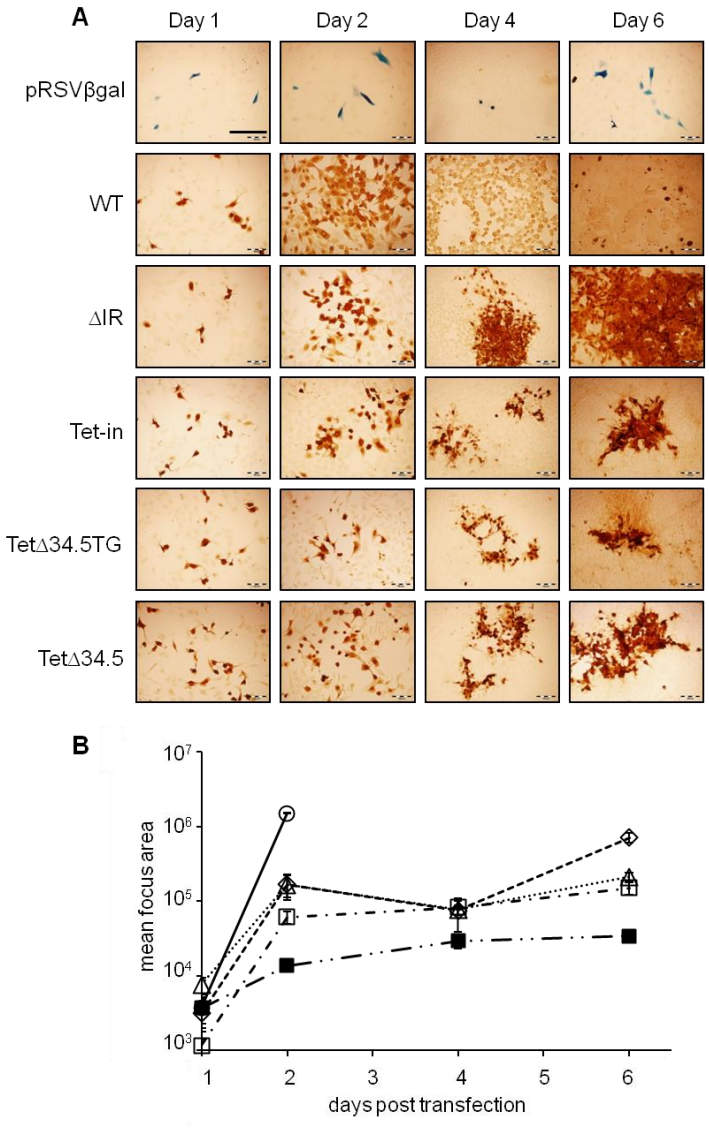

Figure 11. Spread of infection in Vero cells transfected with WT, ΔIR or TetICP0 mutants in semi-solid medium.

A. Vero cells were transfected with DNA (as indicated to the left of each row) encoding the TetICP0 mutants (Tet-in, TetΔ34.5TG or TetΔ34.5), control viruses (WT or ΔIR) or the control plasmid pRSVβgal, which marks transfected cells by β-galactosidase expression. Cells were fixed on various days following transfection (indicated above each column) and stained with anti-HSV-1 antibody (WT, ΔIR and TetICP0 mutants) to detect virus-producing cells, or with 5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside (pRSVβgal), as a control for the apparent increase of transfected cells merely due to cellular duplication. Scale bar: 100 μm.

B. Quantification of mean infection focus area at 1, 2, 4 and 6 days after transfection of WT (solid line, circles) and each mutant: ΔIR (dashed line, diamonds); Tet-in (dashed/dotted line, squares), TetΔ34.5TG (dashed/double dotted line, solid squares) and TetΔ34.5 (dotted line, triangles). Error bars represent the standard error from analysis of 5-12 foci for each data point.

5.4. Simultaneous deletion of *ICP34.5* and TAATGARAT motif abolishes viral replication

Since transfection of TetΔ34.5TG BAC DNA yielded very small plaques which eventually became overgrown by nonproductive cells, I was unable to obtain viral stocks of mutant TetΔ34.5TG in multiple experiments. Cell lysates prepared at different time points revealed negligible infectious particles of TetΔ34.5TG (**Figure 12A** and **12C**). Additionally, when checked for amplicon packaging ability, the TetΔ34.5TG mutant displayed highly reduced efficiency compared to WT, ΔIR and other TetICP0 mutants (Tet-in and TetΔ34.5) (**Figure 12B**).

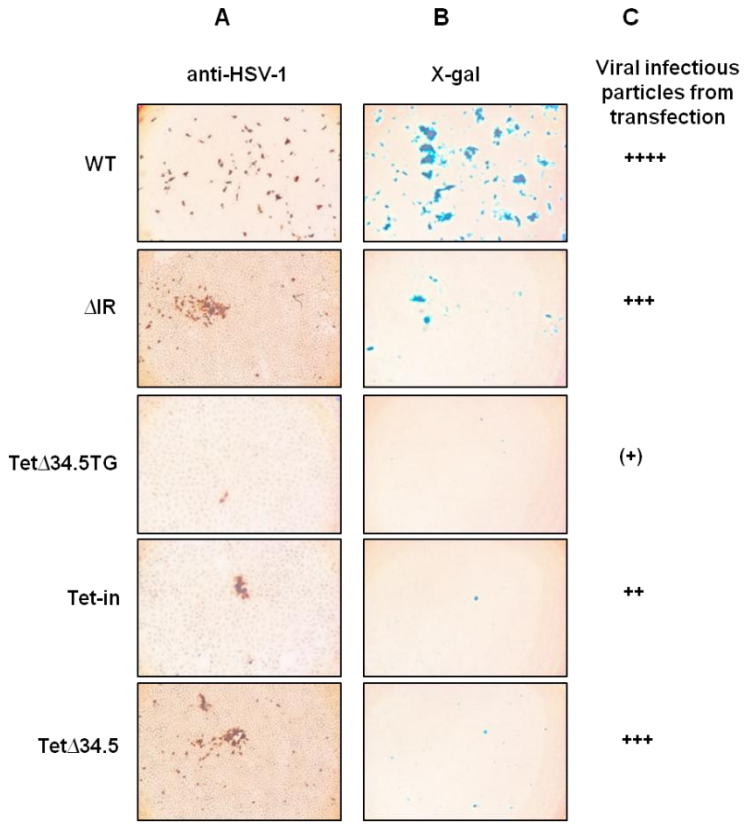

Figure 12. Effect of ICP0 promoter mutations on infectious particle production and amplicon packaging efficiency.

A. Vero cells were transfected with DNA encoding the TetICP0 mutants (Tet-in, TetΔ34.5TG or TetΔ34.5) or control viruses (WT or ΔIR). At 48 hours post transfection, virus was prepared, plated over fresh Vero cells which after 24 hours were stained with anti-HSV-1 antibody to detect the presence of infectious particles. Representative images of these infectious particle assays for each viral mutant are shown.

B. Vero cells were co-transfected with pHSVlac amplicon and DNA encoding the TetICP0 mutants (Tet-in, TetΔ34.5TG or TetΔ34.5) and control viruses (WT or ΔIR). At 48 hours post transfection, virus was prepared, plated over fresh Vero cells which after 24 hours were stained with 5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside (X-gal) to detect the presence of packaged amplicons. Representative images of these amplicon packaging assays for each virus are shown.

C. The plus (+) signs in the right column indicate growth of each mutant in Vero cells. Mutant TetΔ34.5TG exhibited slow formation of HSV-1 antigen positive foci after transfection of BAC DNA (represented as plus (+) sign enclosed in brackets) but all attempts to propagate it were unsuccessful.

These results suggest that the viral infection spread (see section 5.3.2) does not necessarily reflect successful establishment of productive infection. Moreover, negligible packaged virions were obtained for the TetΔ34.5TG mutant. The inability of the TetΔ34.5TG mutant to yield increasing titers of virion particles is not merely due to deletion of the *ICP34.5* gene since transfection of the control construct TetΔ34.5 yielded viral growth similar to ΔIR. Productive infection could not be achieved even by transfection of the TetΔ34.5TG mutant into reverse tetracycline responsive transactivator (rtTA) expressing Vero (Vero-rtTA) cells, indicating that the deletion in TetΔ34.5TG results in a defect in ICP0 expression which cannot be compensated for by the tetracycline-regulated transactivation system.

Next, complementation of the replication defect in TetΔ34.5TG mutant was attempted by (1) co-transfecting the mutant BAC DNA with a plasmid expressing ICP0 (pAXKS) in Vero cells or (2) performing the transfection in human osteosarcoma cell line U2OS. Both of these strategies failed to rescue TetΔ34.5TG mutant replication and negligible yields of infectious particles were obtained. Failure of TetΔ34.5TG to give measurable titers in the U2OS cell line which is known to support growth of ICP0 null mutants (Yao & Schaffer, 1995) suggests that in the absence of ICP34.5, ICP0 executes an essential function which is not complemented by the U2OS cell line.

To test if growth defects in TetΔ34.5TG are due to the deletion of the consensus TAATGARAT motif, another mutant TetΔTG was generated which contains the *ICP34.5* gene and all the ICP0 promoter elements but not the consensus TAATGARAT motif, which was substituted by 2 tetO repeats (2X tetO, **Figure 13A**). The 2X tetO instead of 7 tetO repeats (as used in TetΔ34.5TG and the other TetICP0 mutants) was used since this has been shown to be equally functional in earlier studies (Marzio et al, 2001; Verhoef et al, 2001) and the smaller size facilitated construction. In Vero cells or the Vero-rtTA cell line, transfection of TetΔTG BAC DNA results in productive infection and packages infectious particles (**Figure 13C**) with more growth in Vero-rtTA cells than in Vero cells (data not shown). The results indicate that mutant viruses constructed in the background of the ΔIR mutant are able to grow when either ICP34.5 (mutant TetΔ34.5) or the ICP0 TAATGARAT motif (mutant TetΔTG) are mutated, but fail to replicate when mutations are made simultaneously in both.

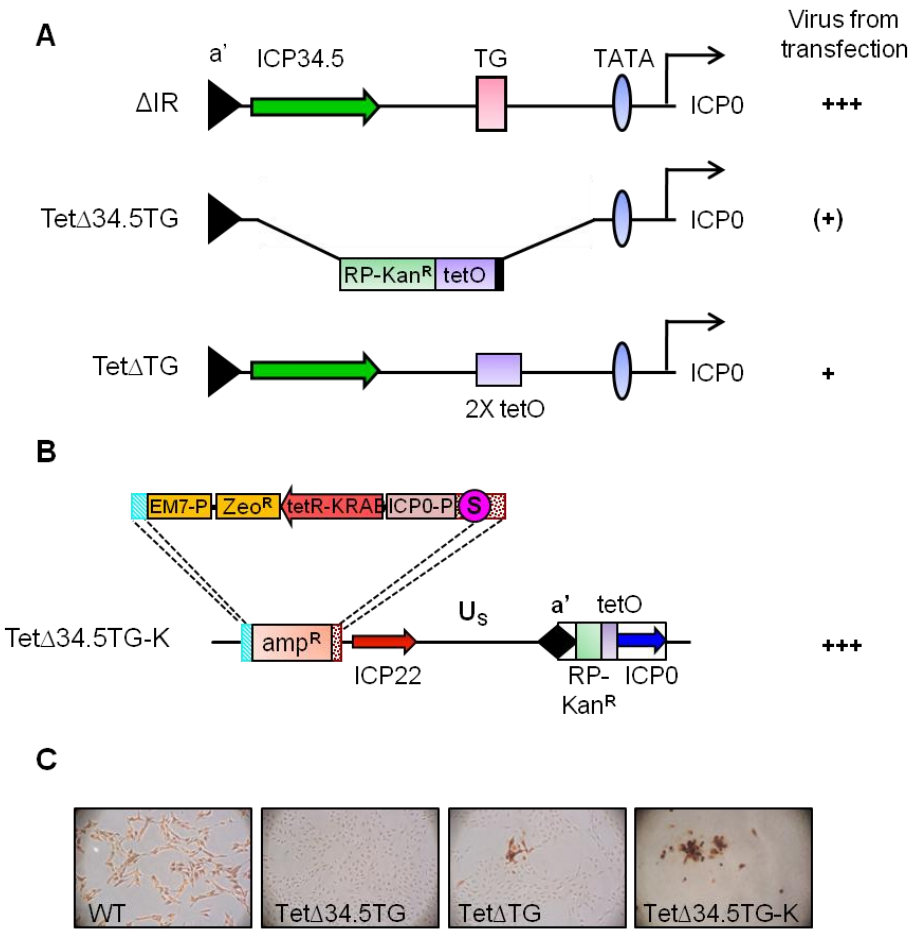

Figure 13. Effect of the *ICP34.5* gene, the ICP22 promoter and oriS on TetΔ34.5TG replication.

A. Diagram of the HSV-1 terminal repeat (TR) region showing the *ICP34.5* gene, the consensus TAATGARAT motif (TG) and the TATA box (TATA) upstream of the transcription start site (rightward arrow) of the *ICP0* gene, all of which are present as a single copy in the ΔIR mutant. In the TetΔ34.5TG mutant, the RP-Kan^R/tetO cassette replaced the whole region encompassing the *ICP34.5* gene up to and including the TG motif. The RP-Kan^R/tetO cassette consists of: the *E. coli* ribosomal S12 (*rpsL*) gene (RP) which results in streptomycin sensitivity; the aminoglycoside phosphotransferase (*aph*) gene which confers kanamycin resistance (Kan^R); target binding sites for the *E. coli* tetracycline repressor consisting of 7 *tet* operator (tetO) sequences. In the TetΔTG mutant, the consensus TAATGARAT sequence was replaced by the RP-Kan^R cassette and 2 repeats of tetO sites (2X tetO).

B. In the TetΔ34.5TG-K mutant, a linear fragment containing the tetracycline repressor-Krüppel-associated box domain fusion protein (tetR-KRAB) and the *Sh ble* gene conferring zeocin resistance (Zeo^R) was inserted to replace the beta-lactamase (*bla*) gene conferring ampicillin resistance (amp^R) of TetΔ34.TG. Transcription of the *Sh ble* gene was driven by the synthetic prokaryotic EM7 promoter (EM7-P) derived from the bacteriophage T7 promoter while the tetR-KRAB transgene was controlled by the ICP0 promoter (ICP0-P), an immediate early promoter of HSV-1. This mutation also inserts an origin of replication (oriS) and reconstitutes the ICP22 promoter in the mutant genome. The open boxes on the right side represent the terminal repeat (TR) region of HSV-1 showing the packaging signal (a'), the RP-Kan^R/tetO cassette and the *ICP0* gene.

The plus (+) signs in the right column indicate varying degrees of growth of each mutant in Vero cells.

C. Vero cells were transfected with DNA encoding WT, TetΔ34.5TG, TetΔTG and TetΔ34.5TG-K. At 48 hours post transfection, virus was prepared and plated over fresh Vero cells which after 24 hours, were stained with anti-HSV-1 antibody to detect the presence of infectious particles. Representative images of the infectious particle assays obtained for each viral mutant are shown.

In addition to *ICP34.5*, *ICP0*, *ICP4*, packaging signal and oriS, the IR region of HSV-1 also contains part of the promoter of *ICP22* gene which is deleted in the ΔIR mutant. Owing to this mutation the ΔIR and ICP0 mutants constructed on the ΔIR backbone may be defective in ICP22 expression. Observations from another set of experiments showed that the defect in TetΔ34.5TG replication was overcome when the mutant genome was modified by reconstituting the ICP22 promoter and inserting an extra copy of the origin of replication (oriS). The generated mutant TetΔ34.5TG-K (**Figure 13B)** produces infectious particles (**Figure 13C**) and could be grown up to titers similar to TetΔ34.5 (data not shown). This mutation was part of a separate set of experiments where I inserted the tetracycline repressor-Krüppel-associated box (KRAB) domain fusion proteins (tetR-KRAB) (Deuschle et al, 1995; Margolin et al, 1994) into the TetICP0 mutant genomes (Tet-in, TetΔ34.5TG and TetΔ34.5) to regulate ICP0 transcription via binding to the tetO sites. The objective of these experiments was to produce an "OFF" state with no viral growth, which can be switched to an "ON" state with efficient lytic replication. The regulatory effects due to the tetR-KRAB fusion protein were not observed in these TetICP0 mutants, but all exhibited enhanced viral replication such that even the modified TetΔ34.5TG mutant became replication-competent.

To test if this rescue of TetΔ34.5TG replication was due to the insertion of oriS or reconstitution of the *ICP22* promoter or both, another mutant was constructed, in which an extra oriS was inserted ectopically at the *loxP* site of the TetΔ34.5TG genome using Cre recombinase-mediated retrofitting (**Figure 14A**) (Kim et al, 1998). Transfection of Vero cells with the generated TetΔ34.5TG-O mutant (**Figure 14A**) containing two copies of oriS (one at TR and another at the *loxP* site) yielded micro-plaques on day 4 (**Figure 14B)**. The plaques however became overgrown by non-productive neighboring cells after a few days and negligible infectious particles were obtained.

These results therefore indicate that simultaneous deletion of both the consensus TAATGARAT motif of the ICP0 promoter and the *ICP34.5* gene disables viral growth (TetΔ34.5TG) in culture and the presence of either of them (as in TetΔ34.5 and TetΔTG respectively) is necessary for maintenance of the viral replication. As the IR deletion also removes the ICP22 promoter, the growth defect in TetΔ34.5TG could also be partially contributed due to the absence of functional ICP22 at immediate early times of infection. Experiments with TetΔ34.5TG-K mutant confirmed that resurrection of the ICP22 promoter and insertion of oriS can rescue the growth defect in TetΔ34.5TG. This rescue is ICP22-dependent as the insertion of oriS alone fails (TetΔ34.5TG-O) to reproduce the effect. However, further experiments need to be done to test if reconstituting the ICP22 promoter alone is sufficient.

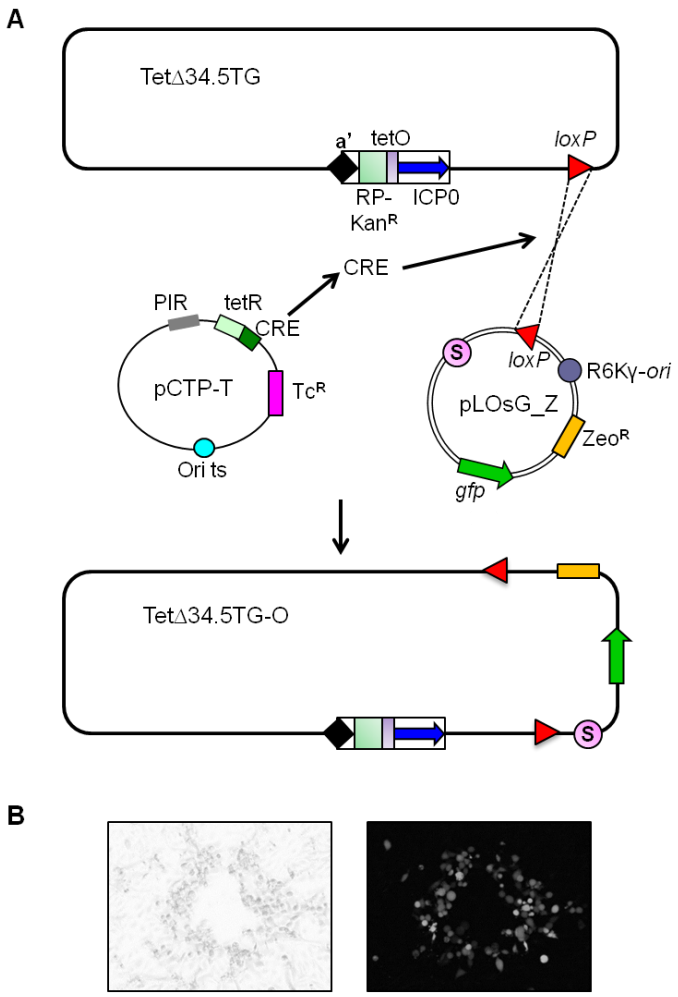

Figure 14. Cre recombinase-mediated insertion of the origin of replication (oriS) in the TetΔ34.5TG mutant.

A. Schematic representation of Cre recombinase-mediated insertion of oriS at the *loxP* site of the TetΔ34.5TG mutant. The shuttle plasmid, pLOsG_Z, contains a *loxP* site, oriS, the green fluorescent protein (GFP) reporter gene (*gfp*), the *Sh ble* gene (which confers zeocin resistance; Zeo^R) and the R6Kγ-*ori* (which enables replication only when π protein is supplied *in trans*). The temperature-sensitive (ts) helper plasmid pCTP-T contains a pSC101-derived thermosensitive replication origin; the Tc^R gene conferring tetracycline resistance sequences for tetracycline-inducible Cre expression, PIR; *pir* gene to supply the π protein to support the replication of the co-introduced plasmids containing the R6Kγ-*ori*. Electrocompetent *E. coli* (DH10B) bacteria containing TetΔ34.5TG BAC DNA were transformed with the pLOsG_Z shuttle plasmid and the pCTP-T helper plasmid. The integrated recombinants (TetΔ34.5TG-O) were selected at 43°C on LB agar plates supplemented with chloramphenicol and zeocin.

B. Vero cells expressing reverse tetracycline responsive transactivator protein (Vero-rtTA) were transfected with bacterial artificial chromosome (BAC) DNA encoding TetΔ34.5TG-O. Representative images of a micro-plaque obtained on day 4 of transfection in bright field (left) and showing GFP autofluorescence (right).

5.5. Mutant TetΔ34.5TG expresses immediate early and late proteins

The ICP0 expression level in TetΔ34.5TG was subsequently analyzed to test if the replication failure of the mutant *in vitro* is due to insufficient availability of ICP0. The expression of a late HSV-1 protein glycoprotein C (gC) was also checked in parallel to uncover if the mutant (TetΔ34.5TG) is able to complete the DNA replication process. BAC DNA encoding the WT or one of the mutant- ΔIR and TetΔ34.5TG genomes was transfected into Vero cells and immunostained with anti-ICP0 and anti-gC antibodies at 8, 24, 32, 48, and 56 hours post transfection. In parallel, the number of anti-HSV-1 immunopositive cells was counted at 8 hours after transfection to normalize the transfection efficiency of the BAC DNAs. **Figure 15A** displays representative images of cells stained with each antibody. Quantification of the immunopositive cells revealed a continuous increase in the number of anti-ICP0 stained cells from 8 hours to 56 hours in WT-transfected culture. Cells transfected with ΔIR and TetΔ34.5TG displayed a similar trend but with values lower than the WT (approximately 4-fold< WT, **Figure 15B**). Anti-gC staining was evaluated in cells 24 hours after transfection with WT or

mutant DNA. Curiously, cells transfected with TetΔ34.5TG were observed immunopositive for gC antigen although with values lower than those of WT and ΔIR (approximately 6-fold< WT and 2-fold< ΔIR) (**Figure 15C**).

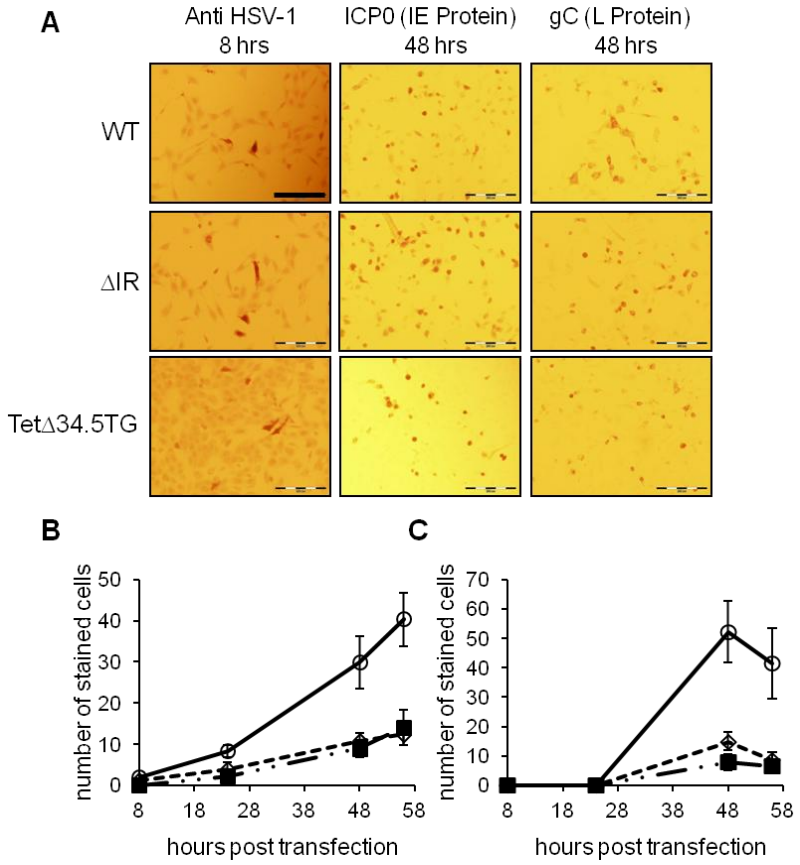

Figure 15. Kinetics of an immediate early (IE) and a late (L) protein in the TetΔ34.5TG mutant.

A. Vero cells were transfected with DNA encoding the mutant TetΔ34.5TG and control viruses (WT and ΔIR). Cells were fixed at 8, 24, 48 and 56 hours post transfection after which they were stained with antibodies specific for the immediate-early (IE) protein ICP0 or the late (L) protein glycoprotein C (gC). As a control for transfection efficiency of the BAC DNAs, the number of immunopositive cells for HSV-1 antigen was counted in parallel transfected samples fixed at 8 hours. Representative images of Vero cells transfected with DNA as indicated to the left of each row and stained with antibody as indicated above each column are shown. Scale bar 100 μm.

B and C. Quantification of the number of immunopositive cells for ICP0 (B) or gC (C) protein in WT (solid line, circles), ΔIR (dashed line, diamonds) or TetΔ34.5TG (dashed/double dotted line, squares)-transfected samples. Error bars represent the standard error from three independent experiments.

ICP0 is a polyprotein which translocates between the nucleus and the cytoplasm of infected cells at different times after infection (Lopez et al, 2001). Cells transfected with TetΔ34.5TG DNA exhibited similar numbers of ICP0 positive cells as those transfected with ΔIR DNA but failed to produce infectious particles, indicating a possibility of defective intracellular localization kinetics of

ICP0 in the mutant. The presence of gC staining, however, indicates that TetΔ34.5TG is able to carry out DNA replication and hindrances to growth occur after that event.

5.6. Viral replication is impaired in ΔIR mutants with ICP0 promoter modifications

Viral stocks of Tet-in and TetΔ34.5 were next prepared to perform a more detailed analysis of the growth of these mutant viruses in Vero cells (**Figure 16A-D**). Using an MOI of 1, growth of the Tet-in mutant was observed to be drastically reduced (**Figure 16A**, approximately 7% of ΔIR titer at 24 hours) while TetΔ34.5 was less affected (**Figure 16A**, approximately 58% of ΔIR titer at 24 hours) but at a lower MOI, growth of TetΔ34.5 was also severely diminished (**Figure 16B**, approximately 5% of ΔIR titer at 24 hours), while Tet-in replication was even more retarded (**Figure 16B**, approximately 0.5% of ΔIR titer at 24 hours).

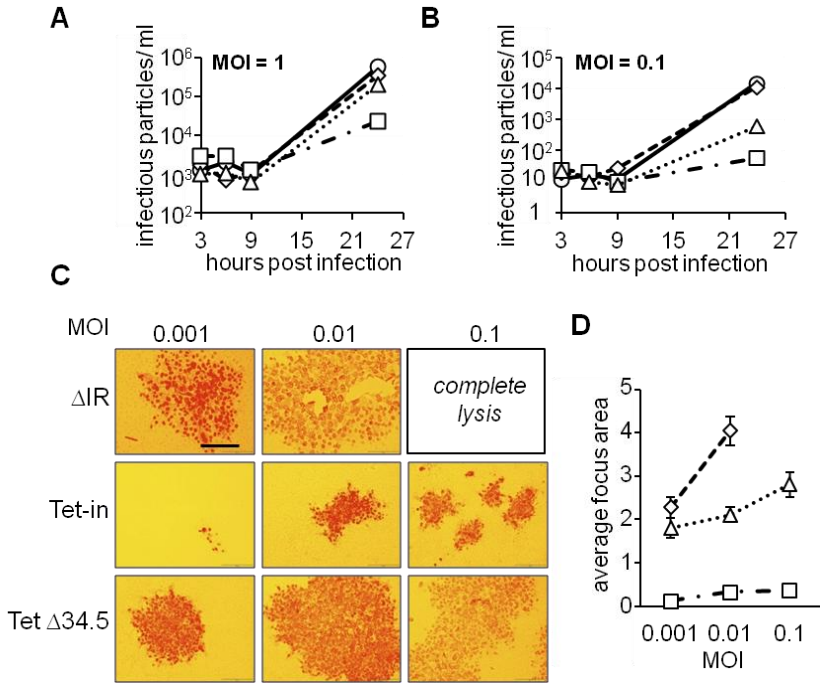

Figure 16. Characterization of the TetICP0 mutants.

A and B. Growth kinetics of wild type HSV-1 (WT) (solid line, circles), the ΔIR mutant (dashed line, diamonds), the TetΔ34.5 mutant (dotted line, triangles) and the Tet-in mutant (dashed/dotted line, squares) in Vero cells infected at a multiplicity of infection (MOI) of 1 (A) and at an MOI of 0.1 (B). The combined intracellular and extracellular titer of infectious particles was measured at 3,6,9 and 24 hours post-infection.

C. Viral spread in Vero cell monolayers overlaid with agar. Cells were infected with each mutant (ΔIR, TetΔ34.5 or Tet-in as indicated to the left of each row) at several MOIs (0.001, 0.01 or 0.1 as indicated above each column), fixed after 48 hours and stained with anti-HSV-1 antibody. Representative infection foci for each sample are shown except for ΔIR at an MOI of 0.1 – all cells were lysed and detached from the plate during the staining process. Scale bar: 200 μm.

D. Quantification of mean infection focus area at the 3 different MOIs for each mutant: ΔIR (dashed line, diamonds); TetΔ34.5 (dotted line, triangles); Tet-in (dashed/dotted line, squares). Error bars represent the standard error from analysis of 7-10 foci for each data point.

Next, viral spread was analysed by infecting Vero cells in semi-solid medium with each mutant at low MOI and examining infection foci after 48 hours (**Figure 16C**). Quantification of the average focus area (**Figure 16D**) revealed that at an MOI of 0.001, infection foci produced by TetΔ34.5 were only slightly smaller than those produced by ΔIR but this lower viral spread of TetΔ34.5 became more evident at higher MOIs. The Tet-in mutant showed even further impairment with much smaller infection foci at all MOIs tested (**Figure 16D**). These results support our hypothesis that ICP0 expression is perturbed in both mutants, since the replication of ICP0 mutants is known to be MOI dependent (Everett et al, 2004; Sacks & Schaffer, 1987).

5.7. VP16 binding to the modified ICP0 promoter using the Tet-ON system or by restoring TAATGARAT proximity increases viral titer

To test whether the reduced growth of Tet-in was a consequence of displacing the TAATGARAT motif by insertion of the RP-Kan^R cassette, the next mutant Tet-inR (**Figure 17A**) was generated using a negative selection strategy to delete the antibiotic marker cassette as described in the Methods. Transfection of Tet-inR DNA yielded virus which grew faster than the parental Tet-in mutant, with plaques appearing after 3 days. To investigate the role of VP16 transactivation of the ICP0 promoter on the replication of these mutants, the viral titers were analyzed at 24 hours after infecting Vero and Vero-rtTA cells at an MOI of 0.1. **Figure 17B** shows that in Vero-rtTA cells, titers of the Tet-in mutant were significantly higher (approximately 2-fold) than in Vero cells, indicating that the inserted tetO sites can compensate for the displaced TAATGARAT motif in this mutant, although not completely to WT levels. Removal of the 1.5 kb displacement in the Tet-inR mutant restored titers in Vero cells to the same level as in Vero-rtTA cells. Titers of the WT virus in Vero and Vero-rtTA cells were indistinguishable, confirming that the observed effect was mediated through the tetO sites. No significant difference in titer was observed by growth of TetΔ34.5 in Vero and Vero-rtTA cells, indicating that the tetO sites in this mutant are probably too distant from the ICP0 TSS to regulate viral replication.

Curiously, although the presence of rtTA significantly increased viral replication of the Tet-in mutant, the presence of doxycycline did not affect the growth of Tet-in or any of the other tetO-containing mutants (**Figure 17B)**. This result indicates that: (1) in the absence of doxycycline, rtTA can mimic VP16-mediated transactivation of ICP0 expression but (2) increased DNA-binding of rtTA in the presence of doxycycline does not offer further advantage for viral replication.

Comparison of the different mutants to WT virus in this experiment revealed a small drop in titer of TetΔ34.5 (down to approximately 24% of WT titer) and a much larger decrease in Tet-in and Tet-inR (down to approximately 2-4% of WT titer). These data indicate that deletion of the *ICP34.5* gene results in a modest reduction of viral titers but that the ICP0 promoter modification and tetO insertion in the Tet-in and Tet-inR mutants significantly impairs viral replication.

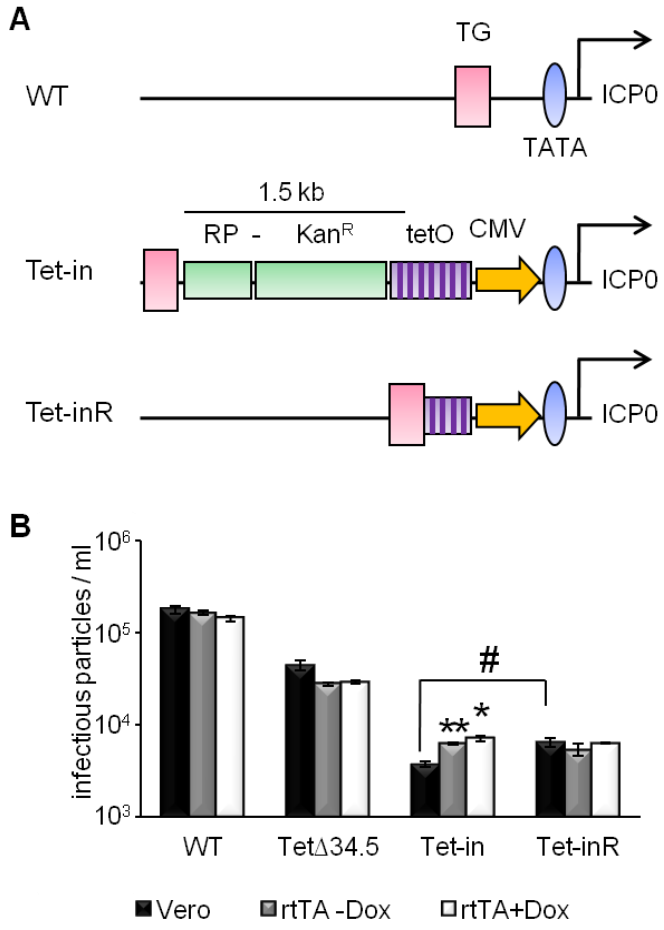

Figure 17. Effect of the reverse tetracycline responsive transactivator (rtTA) on ICP0 promoter mutants with tetO insertions.

A. Schematic representation of the ICP0 promoter region showing the consensus TAATGARAT motif (TG), the TATA box (TATA) and transcription start site (rightward arrow) of the *ICP0* gene in the wild-type HSV-1 (WT) genome. In the Tet-in mutant, the TG motif was displaced 1.5 kilobase (kb) from its original position by insertion of the RP-Kan^R/tetO cassette, composed of: the *E. coli* ribosomal S12 (*rpsL*) gene (RP) which results in streptomycin sensitivity; the aminoglycoside phosphotransferase (*aph*) gene which results in kanamycin resistance (Kan^R); target binding sites for the *E. coli* tetracycline repressor consisting of 7 *tet* operator (tetO) sequences linked to the minimal cytomegalovirus (CMV) immediate early promoter. In the Tet-inR mutant, the TG motif was returned closer to its original position by RP-Kan^R selection cassette deletion, leaving four intact *tet* operator (tetO) sequences.

B. Viral titers of WT and the three mutants TetΔ34.5, Tet-in and Tet-inR at 24 hours after infection at a multiplicity of infection (MOI) of 0.1 in Vero cells, or Vero cells expressing rtTA in the absence of doxycycline (rtTA -Dox) or in the presence of doxycycline (rtTA +Dox). Histogram represents mean values of three independent experiments. Error bars indicate standard errors. Statistically significant increases (Student's two-tailed *t*-test) in titer with respect to the Tet-in titer in Vero cells are indicated by asterisks (** $P < 0.002$, Tet-in titer in rtTA-expressing Vero cells in the absence of doxycycline; * $P < 0.02$, Tet-in titer in rtTA-expressing Vero cells in the presence of doxycycline) and by the hash sign (# $P < 0.05$, Tet-inR titer in Vero cells).

5.8. ICP0 protein levels are abnormally elevated as a result of TAATGARAT displacement in the ICP0 promoter

Next, the effects of the tetO insertions on ICP0 protein expression were analyzed in western blots from cells lysates prepared 24 hours after infection at an MOI of 1 (**Figure 18A**). In all samples, in addition to the 110 kDa band corresponding to full-length ICP0, a 66 kDa band was also observed which has previously been described as due to the major proteolytic cleavage product of ICP0 (Gu et al, 2009). Since multiple factors affect the kinetics of this cleavage, the intensities of both bands were quantified (**Figure 18B** and **18C**) to characterize the effects of the tetO insertions on ICP0 translation as well as processing.

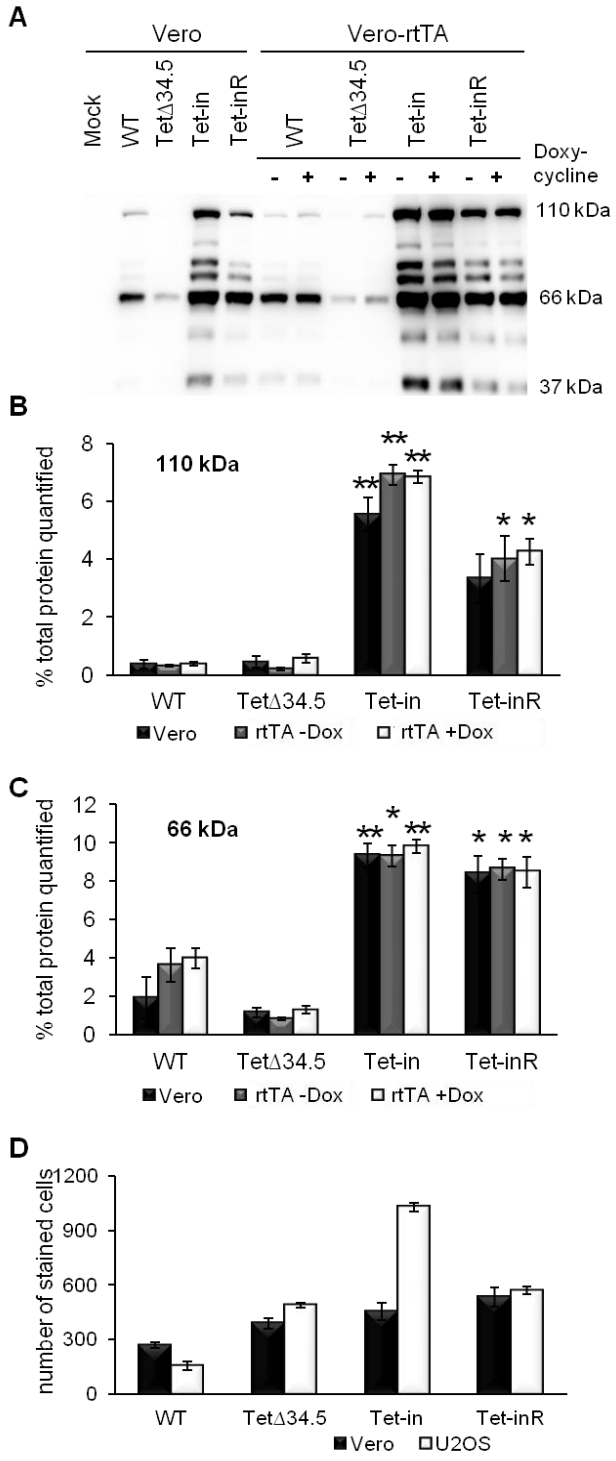

Figure 18. ICP0 protein expression by mutants in the promoter region.

A Western blot of lysates made from Vero cells or Vero cells expressing reverse tetracycline responsive transactivator (rtTA) after infection by wild-type HSV-1 (WT), TetΔ34.5, Tet-in and Tet-inR mutants at a multiplicity of infection (MOI) of 1 for 24 hours. Negative control lysates made from mock-infected cells (Mock) did not exhibit any ICP0-specific immunoreactivity whereas various ICP0 polypeptides could be detected in infected cells, including full-length (110 kDa) ICP0, and major cleavage products of 66 kDa and 37 kDa.

B and C. Histograms representing relative protein quantity of the 110 kDa species (B) and the 66 kDa cleavage product (C) derived by densitometry of the electrophoretic bands shown in A, expressed as a percentage of the total protein quantified. Mean values from three independent experiments are shown, with error bars representing the standard error. Statistically significant differences (Student's two-tailed *t*-test) with respect to the WT values in respective cell lines are indicated by asterisks (** $P < 0.007$; * $P < 0.05$).

D. Viral titers of WT and mutant viruses (TetΔ34.5, Tet-in and Tet-inR) at 24 hours after infection at an MOI of 0.007 in Vero or U2OS cell line. Histogram represents quantification of the number of positive cells for anti-HSV-1 staining in both cell types infected by viral samples. Error bar represents the standard error from three independent experiments.

In Vero cells, both ICP0 bands were detected at similar levels in extracts from the TetΔ34.5 mutant compared to WT virus. An unexpected finding was an abnormally high level of the ICP0 bands (approximately 15-fold > WT for 110 kDa band; 5-fold > WT for 66 kDa band) in the Tet-in mutant. Removal of the 1.5 kb displacement of the TAATGARAT element in the Tet-inR mutant resulted in reduction of full-length ICP0 expression down to intermediate levels (with respect to WT and Tet-in) for both the full-length 110 kDa protein (approximately 9-fold > WT) as well as the 66 kDa cleavage product (approximately 4-fold > WT).

In Vero-rtTA cells, ICP0 expression following infection by WT virus or the Tet Δ34.5 mutant was similar to that observed in Vero cells. However, in the Tet-in and Tet-inR mutants, full-length ICP0 was expressed at slightly higher levels compared to that in Vero but this difference was not significant. Curiously ICP0 expression by the mutants in rtTA cells was unaffected by the presence of doxycycline except for TetΔ34.5, in which full-length ICP0 levels appeared to be higher, although this difference was not statistically significant (**Figure 18B** and **18C**).

Since the titer of all the viruses used in this study was measured in Vero cells, we next investigated if the observed ICP0 overexpression in Tet-in and Tet-inR could be attributed just to the underestimation of infectious particles since plaque formation by ICP0 mutants in Vero cells is known to be MOI dependent and a similar effect may apply to our infectious particle assay, which depends on HSV-1 antigen expression in infected cells. We carried out this assay for WT and ICP0 mutants (Tet-in, Tet-inR and TetΔ34.5) in the U2OS cell line which is known to complement ICP0 mutants (Yao & Schaffer, 1995) and the obtained titers were compared those measured simultaneously in Vero cells. The histogram in **Figure 18D** displays the titers measured in both the cell types when infected at low multiplicity (MOI 0.007). All the viruses showed similar infectious particle counts in both Vero and U2OS cell lines except the Tet-in mutant which displayed almost double the titer in U2OS compared to that of Vero cells. Normalization of the ICP0 quantification obtained in western blot using these titers measured in U2OS cells revealed that ICP0 levels were still elevated in the Tet-in mutant (approximately 7-fold > WT for the 110 kDa band) indicating that this effect was not merely due to infection of the mutant virus at a higher MOI compared to WT.

To better understand the effect of the mutations on ICP0 expression kinetics, ICP0 level was measured in Vero cells infected for different times with WT, Tet-in and Tet-inR mutant at an MOI of 1. The results revealed that the level of full length ICP0 (110 kDa) increased continuously from 8 hours to 48 hours post-infection in all the viruses (**Figure 19A** and **19B**). However, the peak level of the cleaved products (66 kDa and 37 kDa) in WT was observed at 32 hours after which the level decreased, possibly due to degradation of the cleaved products (Gu et al, 2009) (**Figure 19A** and **19B**). In contrast, the expression of the 66 kDa cleaved product in Tet-in and Tet-inR infected cells appeared to increase continuously from 8 hours to 48 hours whereas the peak expression of 37 kDa cleaved product was achieved earlier than WT (**Figure 19A** and **19B**).

Taken together, these results suggest that not only ICP0 protein levels are elevated abnormally in the Tet-in and Tet-inR mutants but that the kinetics for ICP0 cleavage and degradation are also altered.

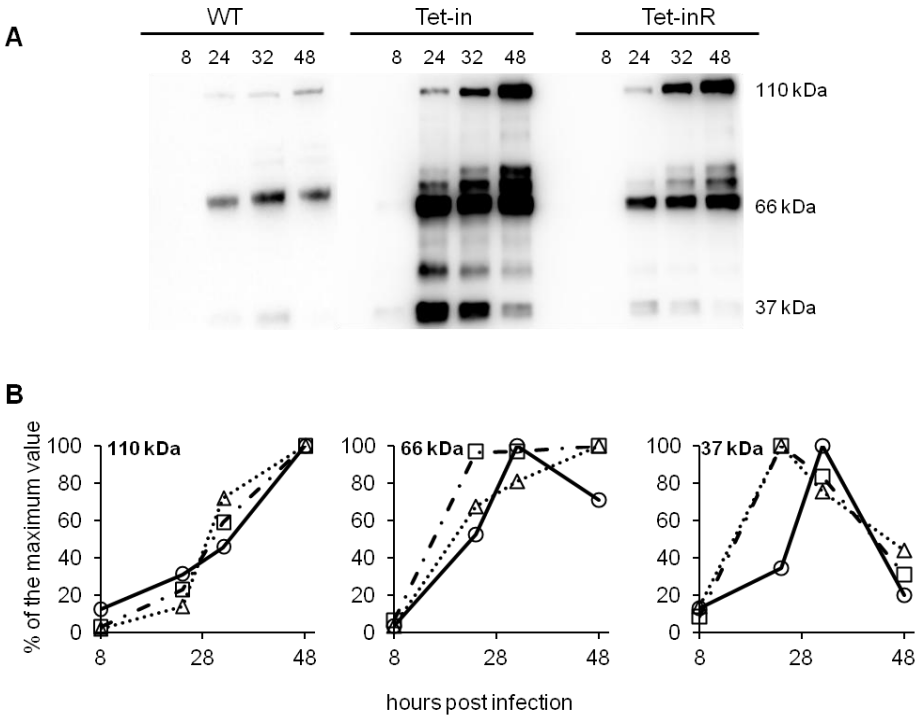

Figure 19. Kinetics of ICP0 protein expression by WT virus and ICP0 promoter mutants.

A. Western blot of lysates made from Vero cells after infection by wild-type HSV-1 (WT), Tet-in and Tet-inR mutants at a multiplicity of infection (MOI) of 1 for 8, 24, 32 and 48 hours (as indicated on the top of the blot). Various ICP0 polypeptides could be detected in infected cells, including full-length (110 kDa) ICP0, and major ICP0 cleavage products of 66 kDa and 37 kDa.

B. Graphs representing protein expression level as a percentage of the maximum value of full-length ICP0 (110 kDa) and the cleavage products (66 kDa, 37 kDa) derived by densitometry of the electrophoretic bands shown in A: WT (solid line, circles), Tet-in (dashed/dotted line, squares) and Tet-inR (dotted line, triangles).

5.9. Single cell analysis confirms elevation of ICP0 protein level in the Tet-in mutant

As an alternative approach to test whether ICP0 levels in Tet-in and Tet-inR infected cells are altered with respect to WT levels; we infected Vero cells with WT virus and the ΔIR, TetΔ34.5, Tet-in and Tet-inR mutants at high dilution (MOI of 0.007) and analyzed the samples after 24 hours by immunocytochemistry using an anti-ICP0 antibody (**Figure 20A**). Fluorescence microscopy analysis of all samples was performed during the same session and photographs were all taken at the same settings. Pixel density quantification revealed that ICP0 expression in single cells infected by Tet-in and Tet-inR was significantly higher than in those infected by the control viruses WT and ΔIR (approximately 2-fold > WT; 3-fold > ΔIR) (**Figure 20B**). The ICP0 level in cells infected by TetΔ34.5 was also slightly higher than that measured in cells infected by ΔIR (**Figure 20B**), but this difference was not significant. Thus, our data at a single cell level confirm our biochemical results

that mutants containing a displacement in the TAATGARAT element (1.5 kb in the Tet-in mutant and 350 bp in the Tet-inR mutant) exhibit increased ICP0 expression.

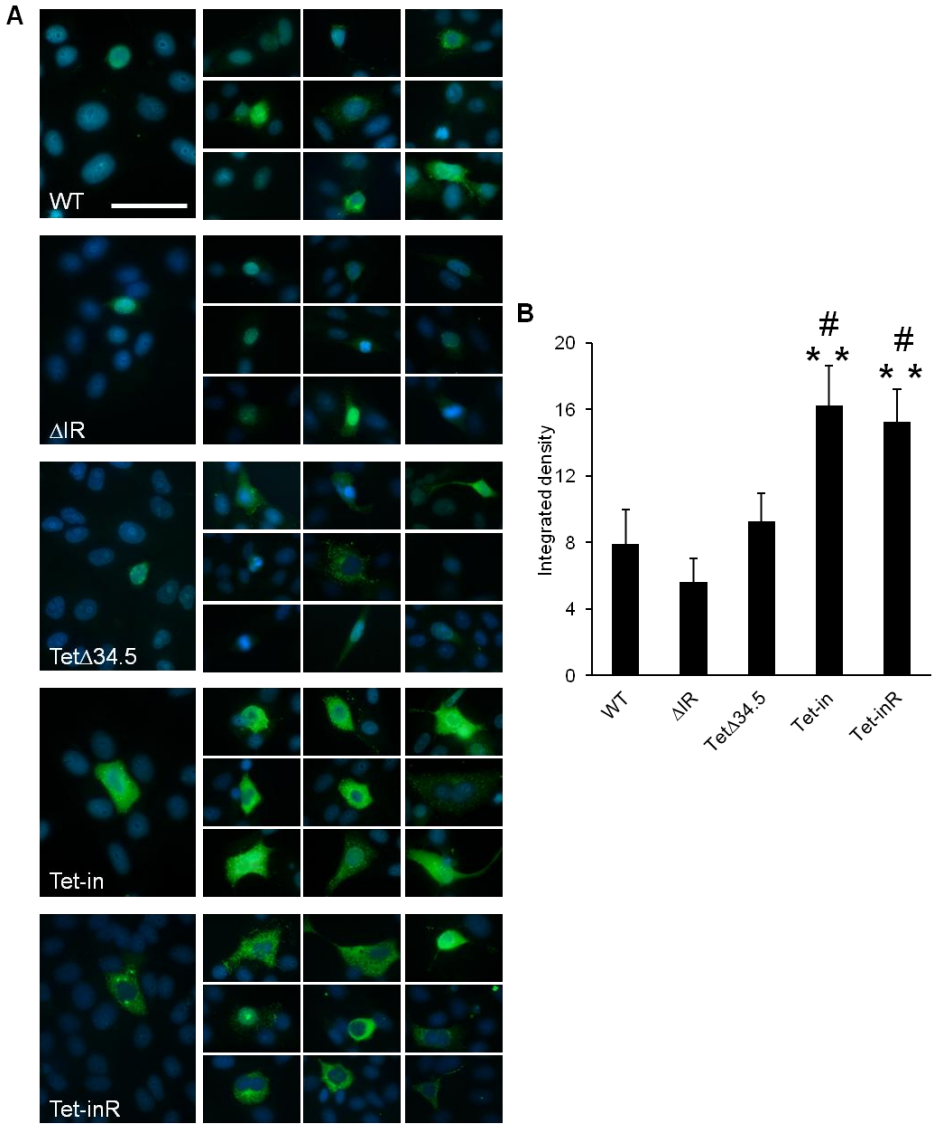

Figure 20. Immunocytochemistry of Vero cells infected by wild type HSV-1 (WT), ΔIR and TetICP0 mutants (TetΔ34.5, Tet-in and Tet-inR) at a multiplicity of infection (MOI) of 0.007 for 24 hours.

A. Images show fixed cells stained with 4', 6-diamidino-2-phenylindole (DAPI, blue) and antibody specific for ICP0 (green). Each panel displays overview of an imaging field (left) and gallery (right) of representative individual infected cells showing ICP0 staining. Scale bar, 50 μm.

B. Quantification of average ICP0 expression per cell. Histogram representing average values obtained from integrated pixel densities of ICP0 staining in selected positive cells for each mutant. Error bars represent the standard error from analysis of 10-15 immunofluorescent positive cells for each virus. Asterisks indicate statistically significant (Student's two-tailed *t*-test) increase in ICP0 expression compared to ΔIR (** $P < 0.0007$) while the hash symbol indicates a significant increase compared to the WT (# $P < 0.02$).

5.10. Translocation of ICP0 in the Tet-in mutant occurs earlier

Another aspect of ICP0 with important functional implications is its intracellular localization. In Vero cells infected by WT virus, newly synthesized ICP0 is principally nuclear where it colocalizes with PML and other ND10 components (Everett & Maul, 1994). Between 5 and 9 hours after infection, ND10 bodies are disrupted and ICP0 is translocated from the nucleus and dispersed in the cytoplasm (Lopez et al, 2001). Since proteasome-dependent degradation of ICP0 has been reported to occur principally in the nucleus (Gu et al, 2009), the high level of ICP0 in cells infected by the Tet-in mutant were examined to find if it might be due to a lower degradation rate because of a more cytoplasmic localization. Vero cells were infected with WT virus, and the TetΔ34.5, Tet-in and Tet-inR mutants at an MOI of 1 and immunostained with anti-ICP0 and anti-PML antibodies at 4, 8 and 24 hours post infection (**Figure 21**).

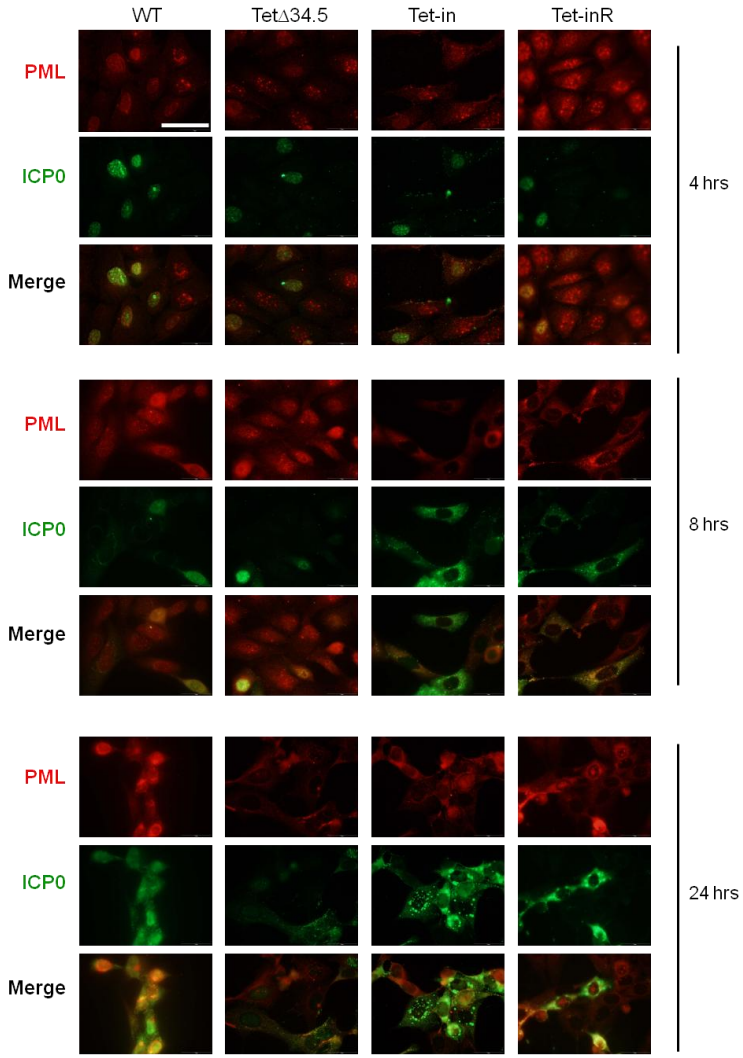

Figure 21. Immunocytochemistry of Vero cells infected by wild type HSV-1 (WT), and TetICP0 mutants at a multiplicity of infection (MOI) of 1 for 4, 8 and 24 hours.

Images show fixed cells stained with antibodies specific for PML (red) and ICP0 (green), and the merge of the red and green channels to visualize colocalization of PML and ICP0. Scale bar, 50 μm.

At 4 hours post infection, cells infected by all viruses exhibited nuclear ICP0 staining that co-localized with PML bodies. At 8 hours post infection, all cells expressing ICP0 exhibited non-punctate PML staining, indicating disruption of ND10 bodies. Mixed nuclear and cytoplasmic ICP0 staining was observed in cells infected by WT virus or the TetΔ34.5 mutant whereas only cytoplasmic ICP0 staining was observed in cells infected by the Tet-in and Tet-inR mutants. At 24 hours post infection, cells infected by all viruses show completely cytoplasmic ICP0 staining (**Figure 21**). Thus, ICP0 in cells infected by Tet-in and Tet-inR translocates to the cytoplasm earlier than in cells infected by WT virus. Additionally, in the case of Tet-in, the protein aggregated in vesicle-like structures in the cytoplasm which could even be observed in lower amounts in cells at 4 and 8 hours post-infection (**Figure 21**).

5.11. Qualitative and quantitative differences of *ICP0* transcription in the Tet-in mutant

To better understand the more direct effects of the modifications introduced in the Tet-in mutant we next studied ICP0 mRNA levels in Vero cells infected by Tet-in at an MOI of 1 compared to those infected by WT and ΔIR virus. Total RNA was prepared from cells 8 and 24 hours post infection and RT-PCR was used for semi-quantitative analysis of ICP0 transcripts (**Figure 22**). Since the inserted tetO cassette contains a minimal CMV promoter which might play a role in increasing ICP0 expression, oligonucleotides were used to detect transcripts originating at both the CMV start site as well as the ICP0 start site. As expected, ICP0 mRNA expressed by WT virus or the ΔIR mutant was derived only from the ICP0 start site (**Figure 22A**) whereas that expressed by the Tet-in mutant also consisted of a subset originating from the CMV start site (**Figure 22A**, middle row, rightmost two lanes). Densitometric quantification of triplicate experiments revealed that ICP0 mRNA levels of WT virus and the ΔIR mutant were similar whereas that of the Tet-in mutant was almost twice as high at 8 hours post-infection, but not significantly higher at 24 hours (**Figure 22B**). Transcription from the CMV start site in the Tet-in mutant showed a significant change from 8 to 24 hours (**Figure 22B** second panel) and although the proportion of these transcripts out of the total ICP0 mRNA cannot be accurately measured by our RT-PCR method, it can be seen that the kinetics of total ICP0 transcription (**Figure 22B** first panel, compare Tet-in to WT) is perturbed by the extra activity from the CMV start site. ICP4 mRNA levels were also analzed in this experiment (**Figure 22A** and **22B** third panel) which exhibited similar kinetics to the ICP0 samples, most likely reflecting the role of ICP0 as a transactivator of IE genes.

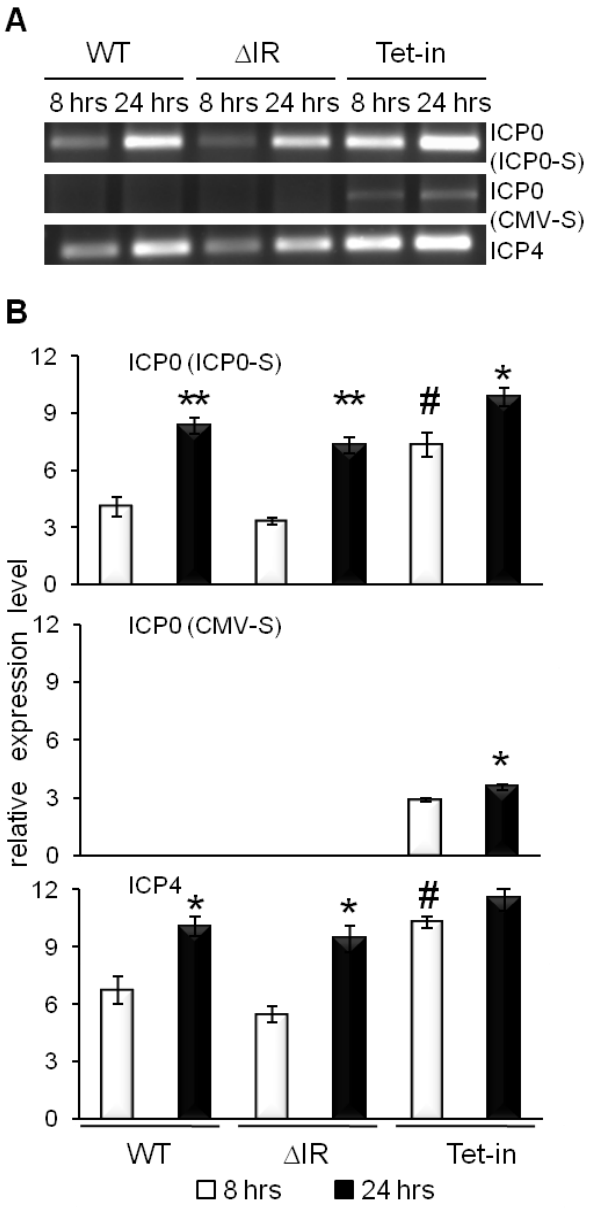

Figure 22. Semi-quantitative RT-PCR analysis of ICP0 mRNA expression by the Tet-in mutant.

Vero cells were infected with the wild type HSV-1 (WT), and the ΔIR and Tet-in mutants at a multiplicity of infection (MOI) of 1 and harvested after 8 or 24 hours for total RNA isolation.

A. Representative images of agarose gel electrophoresis of RT-PCR products using oligonucleotides specific for ICP0 transcripts originating either from the native ICP0 start site (top row, ICP0-S) or the cytomegalovirus (CMV) immediate-early minimal promoter start site (middle row, CMV-S), or ICP4 transcripts (bottom row).

B. Histograms representing relative transcript levels derived by densitometry of the electrophoretic bands shown in A. Mean values from three independent experiments are shown, with error bars representing the standard error. Asterisks indicate statistically significant (Student's two-tailed *t*-test) increases in expression at 24 hours with respect to the 8 hour value for the same virus (** P <0.005; * P < 0.05) while the hash symbol indicates a significant difference in the Tet-in sample at 8 hours compared to the WT or the ΔIR controls at 8 hours (# P < 0.05).

5.12. Tet-in and Tet-inR mutants exhibit reduced initial levels of ICP0

The results described above indicate that ICP0 mRNA and protein levels were higher in cells infected by the Tet-in mutant than those infected by WT virus were puzzling in view of the fact that the modification in this mutant would be expected to weaken VP16 transactivation of ICP0 due to the 1.5 kb displacement of the TAATGARAT motif away from the promoter. The present study hypothesized that all of the measurements might reflect delayed events in ICP0 expression kinetics and that perhaps reduced VP16 transactivation might only be directly observable during the immediate-early phase of infection. Thus, an established procedure using chemical inhibitors was next employed to examine HSV-1 gene expression corresponding to immediate-early (IE), early (E) and late (L) phases (**Figure 23** and **24**). Vero cells infected by the test viruses were incubated with cycloheximide for 6 hours to inhibit *de novo* translation, permitting only IE viral mRNA to

accumulate. The cells were then released from the translation block by changing into fresh medium containing: (1) the transcriptional inhibitor actinomycin D (Reich et al, 1961), allowing only the expression of viral IE genes; (2) the DNA synthesis inhibitor acyclovir (Harmenberg & Wahren, 1982), allowing the expression of IE and E, but not L genes; no inhibitor, in which case viral genes of all 3 kinetic classes are expressed (**Figure 23A**).

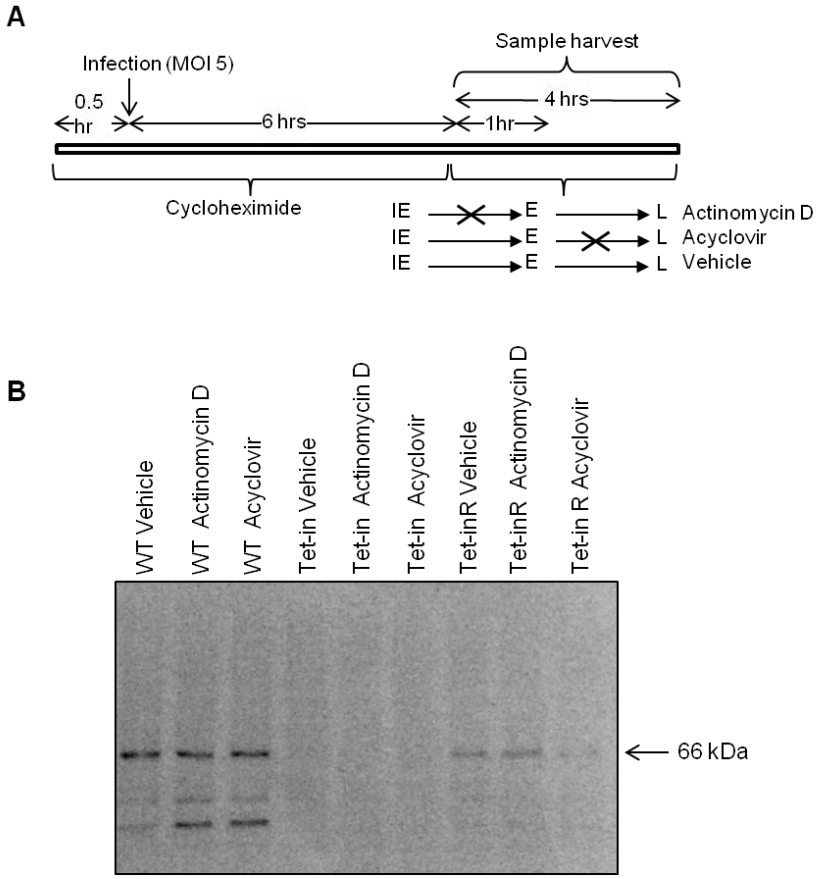

Figure 23. Analysis of ICP0 protein expression kinetics in Tet-in and Tet-inR mutants using pharmacological inhibitors.

A. Schematic representation of the experimental set up. Vero cells were inoculated with the wild type HSV-1 (WT), Tet-in and Tet-inR mutants at a multiplicity of infection (MOI) of 5, treated with cycloheximide 0.5 hours before, to 6 hours after infection, and released thereafter into medium containing actinomycin D, acyclovir, or vehicle only. Total protein was extracted from the cells at 4 hours after release from cycloheximide and analyzed by western blot.

B. Representative image of western blot using an ICP0-specific antibody showing detectable levels of the 66 kDa cleavage product of ICP0 in WT and Tet-inR samples. No ICP0 protein expression was detectable in cells infected with the Tet-in mutant nor was full-length ICP0 detectable in any of the samples even at long exposures.

Western blots of protein extracts made from cells 1 and 4 hours after the release from cycloheximide treatment revealed very low levels of ICP0; only the 66 kDa fragment could be detected at 4 hours, and only after long exposure times (**Figure 23B**). Thus RT-PCR was used to

analyse total RNA prepared from the same cells since the sensitivity of this assay is higher. As can be seen from **Figure 24A**, ICP0 expression levels of the Tet-in mutant are reduced in the IE and E phases (**Figure 24B**, asterisks indicate significant decrease of Tet-in samples treated with actinomycinD or acyclovir compared to WT samples treated with the same drugs), and as expected, are restored towards WT levels in the Tet-inR mutant (**Figure 24B**, cross-hatch indicates significant increase of vehicle-treated Tet-inR samples compared to vehicle-treated Tet-in samples). ICP0 levels in vehicle-treated Tet-inR samples at 1 hour treatment appeared higher than vehicle-treated WT samples (**Figure 24B**, difference not statistically significant), possibly due to the activity of the CMV promoter.

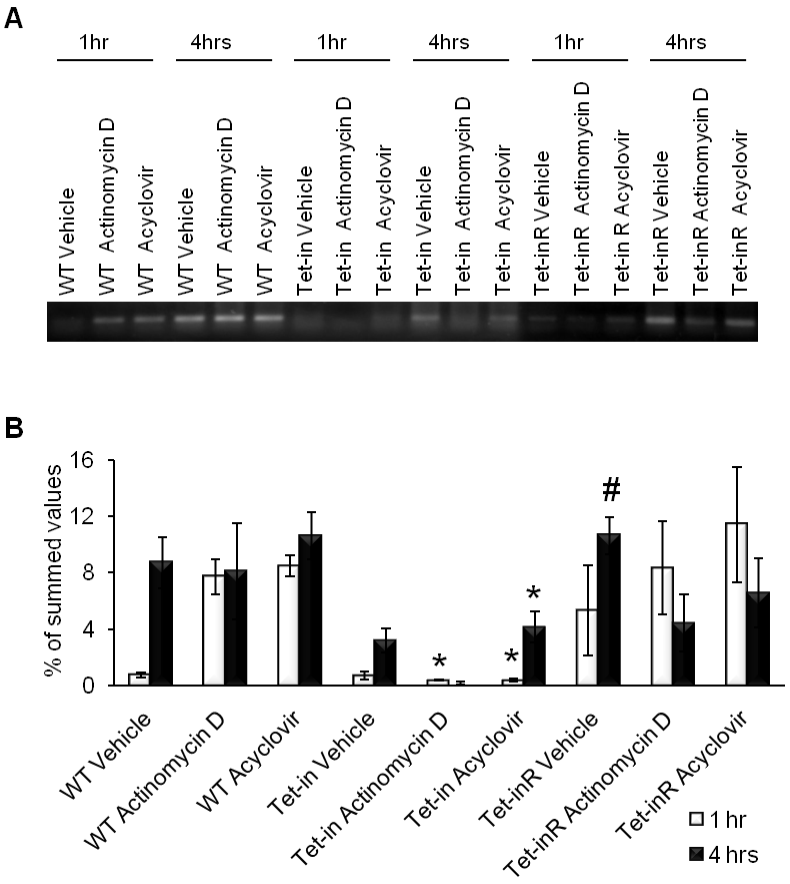

Figure 24. Analysis of ICP0 expression kinetics in Tet-in and Tet-inR mutants using pharmacological inhibitors.

A. Vero cells were inoculated with the wild type HSV-1 (WT), Tet-in and Tet-inR mutants at multiplicity of infection (MOI) 5, treated with cycloheximide 0.5 hours before, to 6 hours after infection, and released thereafter into medium containing actinomycin D, acyclovir, or vehicle only. Total RNA was extracted from the cells at 1 and 4 hours after release from cycloheximide and analyzed by RT-PCR. Representative image of agarose gel electrophoresis of RT-PCR products using oligonucleotides specific for ICP0 transcripts originating from the native ICP0 start site is shown.

B. Histograms representing relative transcript levels derived by densitometry of the electrophoretic bands shown in B. Mean values from three independent experiments are shown, with error bars representing the standard error. Asterisks indicate statistically significant (Student's two-tailed *t*-test) differences in expression with respect to the WT value (* $P < 0.05$) while the hash symbol indicates a significant difference in the Tet-in sample compared to the corresponding Tet-inR sample (# $P < 0.02$).

Thus, these data confirm that the modification of the TAATGARAT site in the ICP0 promoter in the Tet-in mutant diminishes initial ICP0 levels but that this later leads to overexpression due to the characteristics of ICP0 regulatory circuitry.

5.13. Binding of the tetR-KRAB fusion protein to the modified ICP0 promoter reduces Tet-in growth

To investigate the effect of tetR-KRAB-mediated repression on Tet-in replication via the tetO sites inserted in the ICP0 promoter, viral titers were analyzed in Vero and tetR-KRAB expressing Vero (Vero-tetRKRAB) cell lines infected at an MOI of 0.1 for 24 hours. **Figure 25A** shows that in Vero-tetRKRAB cells, the titer of the Tet-in mutant was significantly lower than that observed in Vero cells (down to approximately 25%), indicating that binding of tetR-KRAB to the inserted tetO sites can mediate growth repression, although not completely. Curiously, no effect on viral titer was observed in response to doxycycline. In the next experiment the effect of tetR-KRAB mediated repression was analyzed on ICP0 protein expression level in Vero and Vero-tetRKRAB cell lines infected with Tet-in mutant at an MOI of 0.1.

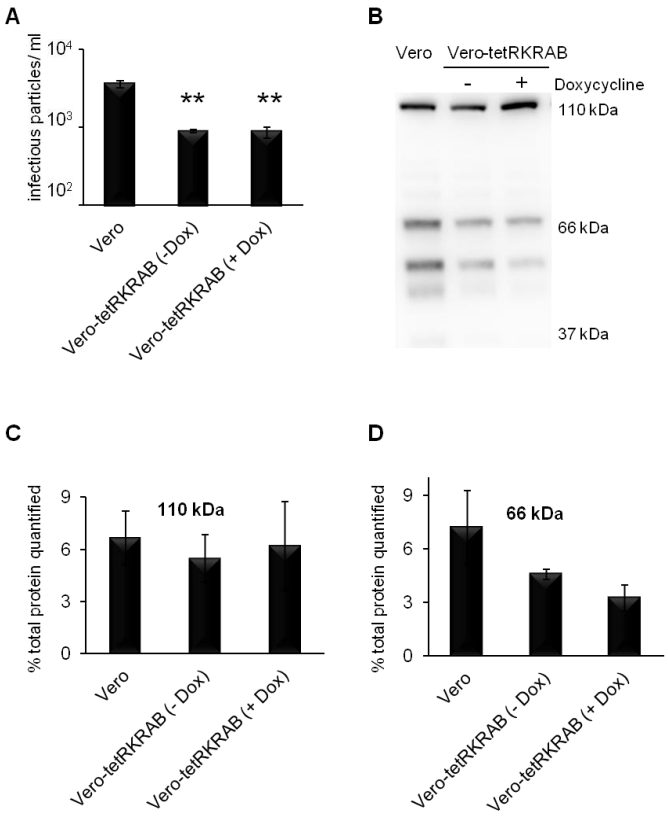

Figure 25. Effect of the tetracycline repressor / Krüppel-associated box domain (tetR-KRAB) fusion protein on the tetO sites inserted in the Tet-in mutant.

A. The viral titer of the Tet-in mutant at 24 hours after infection at a multiplicity of infection (MOI) of 0.1 in Vero, or Vero cells expressing tetR-KRAB protein (Vero-tetRKRAB) in the absence of doxycycline (-Dox) or in the presence of doxycycline (+Dox). The histogram represents mean values of three independent experiments. Error bars indicate standard errors. Statistically significant decreases (Student's two-tailed *t*-test) in Tet-in mutant titer obtained in Vero-tetRKRAB compared to the Vero cells are indicated by asterisks (** $P <$ 0.006).

B. Western blot of lysates made from Vero cells or Vero-tetRKRAB cells after infection by the Tet-in mutant at a multiplicity of infection (MOI) of 0.1 for 24 hours. ICP0 polypeptides were detected in infected cells, including full-length (110 kDa) ICP0, and the major cleavage product of 66 kDa.

C and D. Histograms representing the relative protein quantity of the 110 kDa species (C) and the 66 kDa cleavage product (D) derived by densitometry of the electrophoretic bands shown in B, expressed as a percentage of the total protein quantified. Mean values from three independent experiments are shown, with error bars representing the standard error. No repressive effect of the tetR-KRAB fusion protein was observed on full length ICP0 product (110 kDa); levels of the 66 kDa band were lower in Vero-tetRKRAB cells although this difference was not statistically significant ($P = 0.19$).

Densitometric analysis of ICP0 bands observed in western blots with lysate derived from the two infected cell lines revealed almost negligible effect of tetR-KRAB on full length ICP0 band (110 kDa) (**Figure 25B** and **25C**). However, a reduced level of the 66 kDa band was observed in Vero-tetR KRAB cells (down to approximately 64% of expression in Vero) which decreased further in presence of doxycycline (down to approximately 45% of expression in Vero) (**Figure 25B** and **25D**), although this difference was not statistically significant.

Taken together, these results (**Figure 25**) indicate that tetR-KRAB mediates repressive activity in the Tet-in mutant. However, this effect was incomplete and neither ICP0 expression nor mutant growth was repressed completely. These observations suggest the presence of interference in the system which influences tetO-mediated transcriptional control. As a cautionary note, in these experiments data for control virus (WT or ΔIR) are missing, and thus it is not completely clear if the observed effect is specific to tetR-KRAB and not due to variation between a cell lines.

5.14. ICP0 influences tetracycline-regulated transgene expression and elevates basal transcription from the tetracycline-regulated promoter

To investigate the possibility of interference of HSV-1 IE proteins on tetracycline-mediated transgene regulation, a set of assays using luciferase reporters was designed. A reporter plasmid, pUHC13-3 (Gossen & Bujard, 1992), encoding the *Photinus pyralis* luciferase gene regulated by 7 tetO elements juxtaposed to the cytomegalovirus (CMV) minimal promoter was cotransfected in Vero cells with plasmids expressing tetR-KRAB fusion protein (pLVPRT-tTRKRAB, (Szulc et al, 2006)) and ICP0 (pAXKS) or ICP4 (p175, (Perry et al, 1986)). Plasmid pBluescript II (Alting-Mees & Short, 1989) was used as control nonspecific DNA in all experiments to normalize the total DNA load in each combination of transfection.

Luciferase assays performed at 24 hours post transfection displayed significant doxycycline-responsive repression of tetR-KRAB on tetO regulated luciferase expression, which is perturbed in the presence of the HSV-1 IE proteins ICP0 or ICP4 (**Figure 26A**). The results in this experiment can be summarized as follows: (1) the presence of ICP0 and ICP4 significantly elevates the basal level of luciferase up to 6-fold; (2) interference of ICP0 is greater than that of ICP4; (3) the interference by ICP4 does not completely abolish inducibility and occurs only in the absence of tetR-KRAB; (4) ICP0 produces interference both in the presence and in the absence of tetR-KRAB and (5) the interference was significantly higher in the presence of doxycycline (**Figure 26A**).

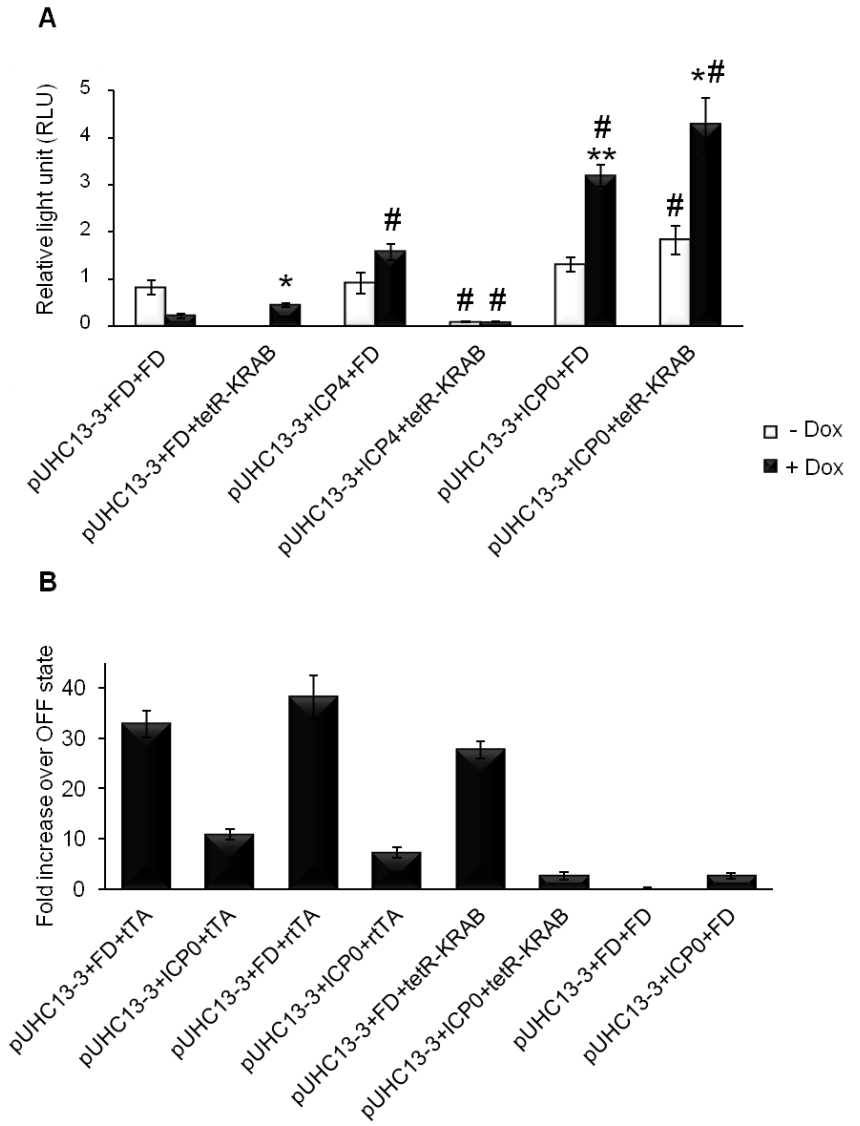

Figure 26. ICP0 interferes with tetracycline-regulated transgene expression.

A. Luciferase expression after co-transfection of a plasmid encoding a tetracycline-regulated luciferase reporter (pUHHC13-3) with plasmids expressing ICP0 or ICP4 and the tetR-KRAB fusion protein. To maintain the total amount of transfected DNA constant in all samples, the plasmid pBluescript II was used as non specific filler DNA (FD). Luciferase activity in the presence (+Dox) and in the absence (-Dox) of doxycycline was determined 24 hours after transfection and represented as relative light units (RLU). Mean values from three independent experiments are shown, with error bars representing the standard error. Asterisks indicate statistically significant (Student's two-tailed *t*-test) differences in expression due to the effect of doxycycline (Dox) (* $P < 0.05$, ** $P < 0.005$) while the hash symbol indicates a significant difference in the value compared to the corresponding control without ICP0 or ICP4 (# $P < 0.05$).

B. Plasmid encoding a tetracycline-regulated luciferase reporter (pUHC13-3) was co-transfected with plasmids expressing ICP0 and the tetracycline-responsive regulators: tTA or rtTA or tetR-KRAB. Plasmid pBluescript II was used as control nonspecific filler DNA (FD) to maintain the total amount of transfected DNA constant in all samples. Luciferase activity in the presence (+Dox) and in the absence (-Dox) of doxycycline was determined 24 hours after transfection. Histogram represents fold increase in the luciferase expression level in ON state over OFF state. Mean values from three independent experiments are shown, with error bars representing the standard error.

These data indicate that expression of the HSV-1 IE genes *ICP4* and *ICP0* elevates basal transcription of the tetO regulated transgene. ICP4 is known to induce transcription by forming a complex with TATA binding protein and transcriptional factor TFIIB (Cheung et al, 1997; Homa et al, 1988). The KRAB domain on the other hand is a DNA binding dependent transcriptional repressor (Margolin et al, 1994) which when tethered to DNA creates a local heterochromatin state extending over a region with a radius of 2-3 kb (Moosmann et al, 1997; Senatore et al, 1999). Binding of the tetR-KRAB protein to the tetO sites hinders RNA polymerase II- mediated transcription and impedes ICP4-mediated transactivation; as observed by a decrease in the interference in the presence of tetR-KRAB. ICP0 also induces transcriptional activation and markedly enhances basal transgene expression. Since, ICP0 has been known to unwind and open up chromatin to increase accessibility to transcriptional machinery (reviewed in (Hagglund & Roizman, 2004)), this could explain why the presence of ICP0 antagonizes tetR-KRAB-mediated functions.

Finally, it was investigated if ICP0 also interferes with the activity of the Tet transactivator proteins- tTA and rtTA. Luciferase assays were performed on the lysates obtained from co-transfection of a plasmid bearing a Tet-regulated transgene reporter (pUHC13-3) with ICP0 and Tet-regulator expressing plasmids (tTA; pUG6SP-tTA or rtTA; pLD-puro-2A-rtTA-TcVA). The values obtained in the assay indicate that tTA and rtTA binding at the tetO sites respectively induce 33- and 38-fold increases in luciferase levels which reduce down to 11- and 7-fold in the presence of ICP0 due to an increase in the baseline luciferase expression level (**Figure 26B**). These results indicate that ICP0 not only interferes with tetR-KRAB-mediated transcriptional repression but also affects tTA and rtTA-mediated transcriptional activation. However, the ICP0-mediated interference on tetR-KRAB is larger than the other tested Tet regulators. In the absence of ICP0, tetR-KRAB represses transgene expression up to 28-fold which reduces approximately to 3-fold in the presence of ICP0 (**Figure 26B)**.

Discussion

6. DISCUSSION

Herpesvirus genomes contain direct or inverted repeats (IR), the reasons for which are not clearly understood. In HSV-1 the duplicated components in the IR and TR sequences include three genes: *ICP4*, *ICP0* and *ICP34.5*. The present study generated the ΔIR mutant using BAC technology with the aim of creating a completely haploid HSV-1 genome to facilitate further genetic engineering. The characterization of ΔIR mutant confirms previous results that IR deletion does not significantly affect viral titer in culture on permissive cells (Brown et al, 1984; Jenkins et al, 1996; Poffenberger et al, 1983) even though there appears to be a slight delay at early time points and in the absence of virion components. Nevertheless, although the double dosage of genes found in IR is not directly necessary for lytic replication, *in vivo* studies have revealed that it is important for HSV-1 pathogenicity (Jenkins et al, 1996; Jenkins & Martin, 1990; Junejo & Brown, 1995), indicating a role in modulation of virus-host interactions. On par with this finding IR deletion studies in other Herpesviruses e.g Equine Herpes Virus-1 (EHV-1) and HCMV have demonstrated no significant effect on viral titer *in vitro* (Ahn et al, 2011; Sauer et al, 2010). However, reduced virulence *in vivo* and lack of genomic isomerisation have been observed to result (Ahn et al, 2011; Sauer et al, 2010).

6.1. A ΔIR mutant deleted in the *ICP34.5* gene and mutated in the ICP0 promoter is deficient in replication

In vitro characterization of mutants constructed over ΔIR backbone reveals that viral replication is severely affected when the remaining copy of the *ICP34.5* gene along with the TAATGARAT elements of the ICP0 promoter is deleted from the ΔIR genome (TetΔ34.5TG mutant). In contrast, replication of the ΔIR genome was not eliminated in separate mutants with deletions either in the *ICP34.5* gene (TetΔ34.5 mutant), the consensus TAATGARAT element (TetΔTG mutant), or in the ICP0 promoter (Tet-in mutant), but combining all modifications resulted in a nonproductive genome (TetΔ34.5TG mutant) which was also severely affected in amplicon packaging ability (**Figure 12**). The TetΔ34.5TG mutant failed to yield measurable viral titers even when transfected into reverse tetracycline responsive transactivator (rtTA) expressing Vero (Vero-rtTA) cells or in human osteosarcoma U2OS cells which are known to support the growth of ICP0 null mutants (Yao & Schaffer, 1995). This suggests that in the absence of ICP34.5, ICP0 executes an essential function which is not either compensated by expression using the tetracycline-regulated transactivation system or complemented by the U2OS cell line. The results from the present study are consistent with earlier findings which demonstrate that although deletion mutants of ICP0 and ICP34.5 are viable in permissive cell lines, they are hypersensitive to host antiviral IFNs (Chou & Roizman, 1992; Mossman et al, 2000) and the two proteins counteract distinct interferon (IFN) barriers to viral transcription and translation (Mossman & Smiley, 2002). Supporting this finding, our ΔIR mutant deleted in the *ICP34.5* gene (TetΔ34.5 mutant) displays a moderate reduction in viral titer

compared to WT at high MOI but this defect was greater at low MOI (**Figure 16**) indicating that the importance of ICP34.5 in viral replication is MOI dependent; at low MOI the synergistic actions of ICP34.5 and ICP0 to target host immune responses are required for productive infection.

The study demonstrates that the inability of the TetΔ34.5TG mutant to produce infectious virions can be overcome by reconstituting the ICP22 promoter and providing an extra origin of replication (TetΔ34.5TG-K; **Figure 13**). However, a subsequent experiment with a mutant carrying an additional oriS ectopically (TetΔ34.5TG-O; **Figure 14**) indicates that reconstitution of the ICP22 promoter has a more important role in complementation of TetΔ34.5TG replication than providing an extra origin of replication (oriS). ICP22 has been previously reported as a critical HSV-1 regulatory factor with roles in alteration of RNA polymerase II, modification of cell cycle components like cdk1, formation of virus-induced chaperone-enriched domains (VICE) and relocalization of host chaperones e.g. Hsc70 (reviewed in (Rice & Davido, 2013)). Despite the fact that ICP22 null mutants replicate efficiently in permissive but not in restrictive cell lines (Poffenberger et al, 1993; Post & Roizman, 1981), it is currently unclear if ICP22 provides any replicative advantage to the virus. One possibility that has been postulated is that ICP22 functions to inhibit antiviral responses and suppresses host gene transcription rather than inducing viral gene expression. Supporting this, earlier studies have shown that several RNA viruses evade the type I interferon system by altering phosphorylation of RNA polymerase II (Thomas et al, 2004; Verbruggen et al, 2011). However, further studies will be required to determine if, in fact, the antiviral response of ICP22 is an important component of its complementation of replication of the TetΔ34.5TG mutant.

Failure of the TetΔ34.5TG mutant in establishing productive infection in spite of expressing IE (ICP0) and L (gC) HSV-1 proteins (**Figure 15**) indicates two points: (1) mere expression of ICP0 is insufficient and correct kinetics of ICP0 processing (nucleus-cytoplasm translocation, proteolytic cleavage) is critical in terms of viral replication; and (2) inhibition of viral growth occurs after completion of L gene expression.

6.2. Distal displacement of the TAATGARAT element from the transcription start site (TSS) leads to overexpression and aberrant processing of ICP0

The present study yielded further insight regarding the dependence of viral replication on ICP0 expression by analysis of the curious properties of Tet-in mutant, a ΔIR-derived virus with an insertion of 7 tetO repeats, displacing the natural TAATGARAT element 1.5 kb away from the TSS. As predicted, viral replication was significantly reduced in this mutant, consistent with the known importance of ICP0 transactivation by VP16 via the TAATGARAT element (reviewed in (Wysocka & Herr, 2003)). However, contrary to expected, western blots revealed an immense overexpression of ICP0 (**Figure 18**). This result was puzzling but support that this was not a technical artifact came from analysis of the Tet-inR mutant, in which I reduced the TAATGARAT displacement from the

TSS from 1.5 kb to 350 bases by deletion of the RP-Kan^R cassette. Relative to the Tet-in mutant, viral replication was improved and ICP0 overexpression was decreased in the Tet-inR mutant and it behaved in a manner intermediate between Tet-in and WT with respect to both viral replication and ICP0 expression (**Figure 17** and **18**). Quantification of ICP0 expression in single infected cells showed significant elevation in ICP0 level in Tet-in and Tet-inR mutants compared to the control viruses (WT, ΔIR) (**Figure 20).** This result verifies that the observed overexpression of ICP0 in TAATGARAT displaced mutants was specifically due to the mutation in the promoter element and not due to possible differences in titer measurements of ICP0 mutants compared to control viruses. As shown in previous studies, ICP0 undergoes proteolytic cleavage during early times into several discrete polypeptides which subsequently degrade in a proteasome dependent pathway (Gu et al, 2009). Western blot performed at different times of infection revealed that ICP0 protein levels in the Tet-in and Tet-inR mutants are not only abnormally elevated but also possess altered kinetics for ICP0 cleavage and degradation (**Figure 19**). Additionally, faster cytoplasmic translocation of ICP0 was observed in Tet-in infected cells which further increases at late times (**Figure 21**). Curiously, a similar phenotype was observed earlier with an ICP4 deletion mutant (d120) (Lopez et al, 2001). As ICP0 has roles in transactivation of other HSV-1 IE genes (Cai & Schaffer, 1992), this study indicates that an alteration in the kinetics of its levels might have pivotal effects on the expression of other IE genes.

Since the dramatic overexpression of ICP0 was the complete opposite of what might be expected by weakening VP16 transactivation, the study postulated that the western blots of ICP0 expression in our study may not detect IE events because protein extracts were made after multiple cycles of non-synchronized viral infection, when dynamic changes in ICP0 expression levels during the course of many single viral replication cycles may mask differences in initial ICP0 levels which would best reflect the direct effects of TAATGARAT displacement in the ICP0 promoter. This was indeed confirmed by the cycloheximide reversal experiment which revealed that IE levels of ICP0 are lower in the Tet-in and Tet-inR mutants compared to WT (**Figure 24**).

The results are consistent with the idea that within the ICP0 promoter, the TAATGARAT element not only mediates IE transactivation, but also forms part of a dynamic control circuit with an inhibitor which normally performs feedback regulation of initial increases in ICP0 expression. Thus, displacement of the TAATGARAT site in the Tet-in mutant results in decreased, and/or delayed inhibition, resulting in an overall (late) augmented level of ICP0 expression. Consistent with this, the TAATGARAT motif has been found to confer both positive and negative responses to cellular factors (Thomas et al, 1998).

6.3. TetO sites inserted in ICP0 promoter cannot substitute for the full activity mediated by the TAATGARAT element

Although growth of the Tet-in mutant observed improved in rtTA expressing Vero cells, viral titer was still well below WT levels, indicating that the artificial tethering of the VP16 transactivation domain to the ICP0 promoter is not sufficient to reproduce the full activation mediated via the TAATGARAT element. One possibility is that other transcription factors are necessary; even though it has been reported that only a small region of VP16 is necessary for complex formation with HCF and Oct-1 (Lai & Herr, 1997), these interactions may be suboptimal with the rtTA chimeric protein. Indeed, interaction with a factor binding to the GC-rich region immediately upstream of the TAATGARAT site in the ICP0 promoter is known to modify transactivation (Kwun & Jang, 2000). A second possibility is that the distance between the TAATGARAT element and the TSS is critical – this is directly indicated by observation that reducing the TAATGARAT displacement in the Tet-inR mutant increased IE transactivation and improved replication, and supported by other work showing that VP16 complexed to HCF and Oct-1 transactivates through TAATGARAT elements only from promoter-proximal positions (Hagmann et al, 1997). Finally, the continuous binding of rtTA to the inserted tetO elements most probably results in transactivation kinetics quite different to that produced by VP16 interaction with the ICP0 promoter during normal viral infection. The above possibilities are not mutually exclusive, and may all contribute to the altered replication of the Tet-in and Tet-inR mutants.

Surprisingly, in rtTA cells, doxycycline did not modify viral replication of any of the tetO-containing mutants (TetΔ34.5, Tet-in or Tet-inR), or produce statistically significant changes in ICP0 protein expression by Tet-in or Tet-inR detectable by western blots. This was not due to anomalies in the tetO cassette since: (1) the tetO repeats were checked by sequencing the BACs encoding these mutants; (2) as mentioned above, increased replication of the tetO-containing mutants was observed in rtTA-expressing cells; (3) in the TetΔ34.5 mutant, although doxycycline did not affect viral replication, in western blots, full-length ICP0 protein levels appeared consistently higher in rtTA cells treated with doxycycline (**Figure 18A**), although this difference was not statistically significant. Since ICP0 is known to act as a promiscuous transactivator by rendering DNA more accessible to transcription factors (Kalamvoki & Roizman, 2010), an explanation for the insensitivity of ICP0 mutants to doxycycline in rtTA cell line is that in the presence of sufficient levels of ICP0, the basal affinity of rtTA for tetO sites is augmented to such an extent that doxycycline does not further augment binding significantly.

6.4. ICP0 interferes with the tetracycline repressor-KRAB (tetR-KRAB)-mediated silencing of tetO-controlled gene transcription

Similar to Vero-rtTA cells, Vero cells expressing tetR-KRAB (Vero-tetRKRAB) did not modify the replication of the Tet-in mutant in response to doxycycline. However, a significant difference between the Tet-in titers obtained in Vero and Vero-tetRKRAB confirms that KRAB repressor activity was mediated by the tetO sites inserted in the mutant. Since in this set of experiment, data for control virus (WT or ΔIR) was missing, it cannot be concluded definitively that the decrease in Tet-in mutant titer in Vero-tetRKRAB cells compared to Vero cell line was exclusively due to the transcriptional repression mediated by KRAB protein and was not due to variation between the two cell lines. The Vero-tetRKRAB cell line used in the experiment was generated by clonal selection and was checked functionally by luciferase assay using a reporter plasmid (data not shown). In view of the fact that the Vero-tetRKRAB cell line displays inducible transcriptional repression of tetO inserted reported gene, the present study suggests that the observed difference in viral titer was specific to the presence of tetRKRAB and not due to variation between the two cell lines, although further experiments would be needed to confirm this.

These results suggest insufficient difference between the ON and OFF state of the tetracycline-regulated switch in our system. Although several HSV-1 derived vectors incorporating tetracycline-regulated switches have been generated (Ho et al, 1996; Schmeisser et al, 2002; Yao et al, 2010; Yao et al, 1998; Yao et al, 2006) some studies indicate that HSV-1 gene products, particularly ICP4, can interfere with the performance of tetracycline-regulated systems (Herrlinger et al, 2000). Indeed, experiments with a reporter plasmid (pUHC13-3) in this thesis demonstrate an increase in basal escape of expression provoked by the presence of ICP0 and ICP4 (**Figure 26A**). ICP0-mediated interference on tetO controlled transcriptional regulation was observed in presence of Tet regulator proteins- tTA, rtTA but maximum interference was observed with tetR-KRAB (**Figure 26B**). The KRAB repressor has been reported to function by closing up chromatin structure (Moosmann et al, 1997; Senatore et al, 1999; Wiznerowicz & Trono, 2003) whereas ICP0 has been found to unwind and open up chromatin to increase accessibility to transcriptional machinery (reviewed in (Hagglund & Roizman, 2004)). This study argues that ICP0 antagonizes KRAB-dependent silencing of tetO-mediated transcription leading to the collapse of the regulatory system.

6.5. Regulation of ICP0 expression in HSV-1 infected cells

ICP0 expression is known to be controlled both by an autostimulatory feed-forward loop as well as an inhibitory feedback loop (Faber & Wilcox, 1986; Lium et al, 1996). Previous studies have indicated that *ICP0* is regulated differently by at least three viral proteins i.e. VP16, ICP4 and ICP0 (Liu et al, 2010). These findings suggest that: (1) VP16 transactivates both the *ICP0* and *ICP4* genes; (2) ICP0 transactivates other IE, E and L genes; (3) ICP4 silences ICP0 transcription when ICP0 fails

to accumulate, and (4) ICP0 itself acts as an inhibitor of ICP4-mediated transcriptional repression of HSV-1 genes, including that of ICP0 itself (Liu et al, 2010) (**Figure 27**).

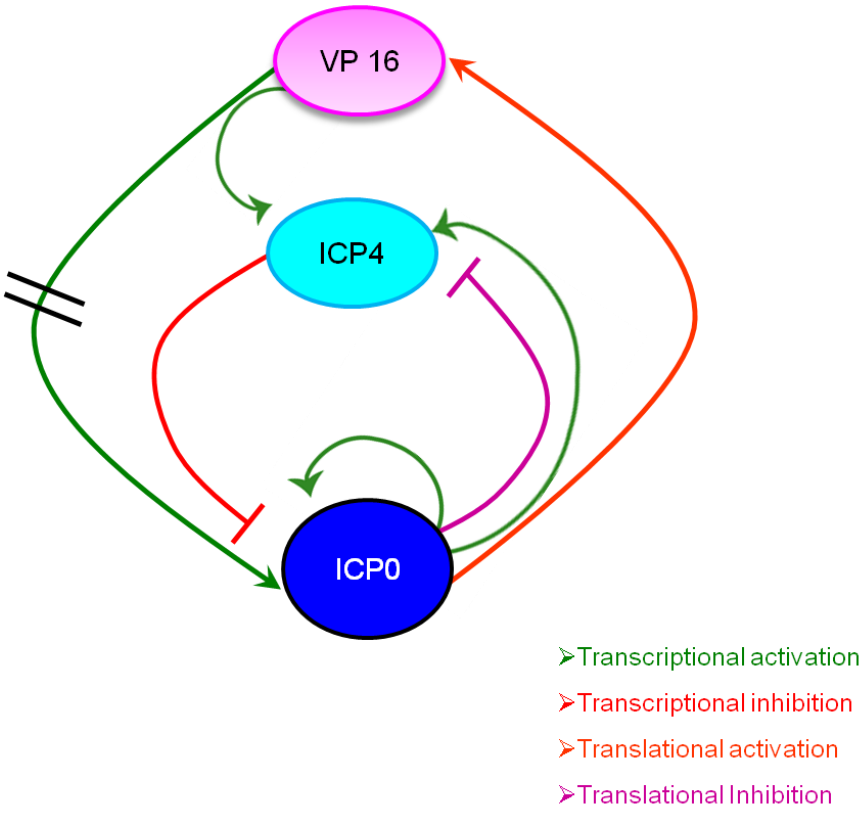

Figure 27. Schematic representation of ICP0 regulatory interactions.

Virion protein 16 (VP16) is a HSV-1 virion protein which induces transcription of immediate early (IE) genes: infected cell protein (ICP) 0 and ICP4. ICP0 transactivates ICP4, as well as its own transcription. When ICP0 fails to accumulate, ICP4 inhibits ICP0 mRNA synthesis but when ICP0 is present it antagonizes ICP4-mediated repression of ICP0. ICP0 also induces late protein VP16 translation. In the present study VP16 mediated transcriptional activation of ICP0 has been interrupted (represented as black double lines).

In the present study I have attempted to substitute the natural VP16-mediated transactivation of *ICP0* with an artificial tetracycline-regulated system to analyze the dependence of viral replication on *ICP0* transcription. The results of this thesis support the role of ICP0 as a critical switch component in HSV-1 productive infection, but also indicate that attempts to impose control over ICP0 expression may not necessarily translate into changes in viral replication. The study concludes that the maintenance of correct ICP0 kinetics is more critical for viral replication than simply switching the expression of the *ICP0* gene ON or OFF. Here, the term "kinetics" refers to dynamic changes in protein expression and processing. ICP0 is a multifunctional polyprotein with complex kinetics, entailing proteolytic cleavage and nuclear to cytoplasmic translocation (Gu et al, 2009; Lopez et al, 2001). The TAATGARAT displacement in Tet-in mutant alters ICP0 kinetics resulting in high levels of ICP0 and faster translocation of the protein to the cytoplasm, all of which result in reduced viral

yield. These results indicate that high levels of ICP0 do not necessarily assure viral growth and may even be detrimental when expression kinetics are altered Additionally, it can also be concluded that the distance between the TAATGARAT element and the transcription start site of ICP0 promoter is critical. The TAATGARAT element imposes complex dynamic control on *ICP0* transcription by mediating not only stimulatory but also inhibitory effects on the promoter. These effects cannot be reproduced by simple tethering of VP16 to the promoter such as with the tetracycline-responsive system. The transcriptional control of *ICP0* may not necessarily translate into changes in viral replication since regulatory feedback mechanisms downstream of ICP0 expression may counteract these changes. Taken together, this would suggest that ICP0 control could more easily be achieved either upstream of the TAATGARAT element, possibly by modifying VP16/Oct-1/HCF complex formation, or at downstream events affecting ICP0 accumulation. Supporting this hypothesis, a recent *in vivo* study has reported that a VP16 mutant virus is incapable of exiting latency (Thompson et al, 2009). On the other hand, an important question will be to determine if wild-type viral titers can be attained by restoring VIC formation or normal ICP0 accumulation.

Many of the HSV-1 genes encoded during lytic replication serve dual functions: (1) assisting in viral replication and (2) counteracting innate or adaptive immune responses. ICP0 and ICP34.5 are particularly important in this regard as they assist in viral replication and target type-1 IFN pathways to circumvent host immune responses ((Mossman & Smiley, 2002), reviewed in (Lanfranca et al, 2014)). ICP34.5 bridges protein phosphatase 1 (PP1) and translation initiation factor (eIF2) via their binding motifs and thereby facilitates the protein synthesis and viral replication (Li et al, 2011). ICP0 is a promiscuous transactivator of viral genes and enhances viral replication both *in vitro* and *in vivo* (Cai & Schaffer, 1989; Everett, 1984; Everett, 1985; Gelman & Silverstein, 1985; Harris et al, 1989; Kalamvoki & Roizman, 2010; Sacks & Schaffer, 1987). In addition, ICP0 disperses ND 10 bodies that are normally associated with transcriptional regulation, growth suppression, and apoptosis. In the present study, complete disruption of viral replication was observed when simultaneous mutations in ICP34.5 and ICP0 (TetΔ34.5TG mutant) were made, indicating that insufficient immunomodulatory activity leads to a block in replication. It will be interesting to test the reversibility of this effect by conditional expression of ICP34.5. In view of the role of ICP0 and ICP34.5 in counteraction of host immune responses, another informative line of research would be to characterize the phenotype of the mutant TetΔ34.5TG in an IFN-deficient cell line.

Conclusions

CONCLUSIONS

1. The inverted repeat (IR) sequences of HSV-1 are dispensable for growth *in vitro*, indicating that a haploid HSV-1 genome is sufficient for viral replication.
2. In the absence of ICP34.5, ICP0 becomes a critical determinant of replication in a haploid HSV-1 genome.
3. The distance between the TAATGARAT element and the transcription start site of ICP0 promoter is critical. An increase in this distance alters ICP0 kinetics, decreasing immediate early expression, but increasing later accumulation of ICP0.
4. Higher levels of ICP0 do not necessarily assure better viral growth and may even be detrimental when expression kinetics are altered.
5. The TAATGARAT element of the ICP0 promoter imposes complex dynamic control on ICP0 expression by mediating both stimulatory and inhibitory effects on transcription. The artificial regulatory tetracycline-responsive system cannot substitute for the full activity mediated by the TAATGARAT element.
6. ICP0 itself interferes with the tetracycline inducible system and therefore cannot be accurately controlled by this system.

CONCLUSIONES

1. La secuencia de la repetición invertida del VHS-1 resulta dispensable para el crecimiento *in vitro*, indicando que un genoma haploide es suficiente para la replicación viral del VHS-1.
2. En la ausencia de ICP34.5, ICP0 es un factor determinante en la replicación del VHS-1 de genoma haploide.
3. La distancia entre el elemento TAATGARAT y el sitio de inicio de la transcripción del promotor de ICP0 es importante. Un aumento en dicha distancia produce una alteración de la cinética de ICP0, disminuyendo la expresión inmediatamente temprana pero, por otro lado, incrementando la acumulación tardía de ICP0.
4. El incremento de los niveles de ICP0 no se correlaciona con un mejor crecimiento viral, incluso pudiendo llegar a actuar en detrimento cuando la cinética de la expresión se haya alterada.
5. El elemento TAATGARAT del promotor de ICP0 ejerce un complejo control dinámico en la expresión de ICP0 mediante la mediación entre los efectos estimulatorios así como inhibitorios de la transcripción. La completa actividad mediada por el elemento TAATGARAT no puede ser sustituida por el sistema artificial regulatorio sensible a tetraciclina.
6. La interferencia de ICP0 con el sistema inducible de tetraciclina impide la regulación de dicho gen mediante este sistema.

Bibliography

Ace CI, McKee TA, Ryan JM, Cameron JM, Preston CM (1989) Construction and characterization of a herpes simplex virus type 1 mutant unable to transinduce immediate-early gene expression. *J Virol* **63**(5)**:** 2260-2269

Ahn B, Zhang Y, Osterrieder N, O'Callaghan DJ (2011) Properties of an equine herpesvirus 1 mutant devoid of the internal inverted repeat sequence of the genomic short region. *Virology* **410**(2)**:** 327-335

Alting-Mees MA, Short JM (1989) pBluescript II: gene mapping vectors. *Nucleic Acids Res* **17**(22)**:** 9494

Baim SB, Labow MA, Levine AJ, Shenk T (1991) A chimeric mammalian transactivator based on the lac repressor that is regulated by temperature and isopropyl beta-D-thiogalactopyranoside. *Proc Natl Acad Sci U S A* **88**(12)**:** 5072-5076

Becker Y, Dym H, Sarov I (1968) Herpes simplex virus DNA. *Virology* **36**(2)**:** 184-192

Bertani G (1951) Studies on lysogenesis. I. The mode of phage liberation by lysogenic Escherichia coli. *J Bacteriol* **62**(3)**:** 293-300

Boehmer PE, Nimonkar AV (2003) Herpes virus replication. *IUBMB Life* **55**(1)**:** 13-22

Bonnerot C, Rocancourt D, Briand P, Grimber G, Nicolas JF (1987) A beta-galactosidase hybrid protein targeted to nuclei as a marker for developmental studies. *Proc Natl Acad Sci U S A* **84**(19)**:** 6795-6799

Boutell C, Orr A, Everett RD (2003) PML residue lysine 160 is required for the degradation of PML induced by herpes simplex virus type 1 regulatory protein ICP0. *J Virol* **77**(16)**:** 8686-8694

Boutell C, Sadis S, Everett RD (2002) Herpes simplex virus type 1 immediate-early protein ICP0 and is isolated RING finger domain act as ubiquitin E3 ligases in vitro. *J Virol* **76**(2)**:** 841-850

Braselmann S, Graninger P, Busslinger M (1993) A selective transcriptional induction system for mammalian cells based on Gal4-estrogen receptor fusion proteins. *Proc Natl Acad Sci U S A* **90**(5)**:** 1657-1661

Brinster RL, Chen HY, Warren R, Sarthy A, Palmiter RD (1982) Regulation of metallothionein--thymidine kinase fusion plasmids injected into mouse eggs. *Nature* **296**(5852)**:** 39-42

Brown SM, Harland J, Subak-Sharpe JH (1984) Isolation of restriction endonuclease site deletion mutants of herpes simplex virus. *J Gen Virol* **65 (Pt 6):** 1053-1068

Cai W, Astor TL, Liptak LM, Cho C, Coen DM, Schaffer PA (1993) The herpes simplex virus type 1 regulatory protein ICP0 enhances virus replication during acute infection and reactivation from latency. *J Virol* **67**(12)**:** 7501-7512

Cai W, Schaffer PA (1992) Herpes simplex virus type 1 ICP0 regulates expression of immediate-early, early, and late genes in productively infected cells. *J Virol* **66**(5)**:** 2904-2915

Cai WZ, Schaffer PA (1989) Herpes simplex virus type 1 ICP0 plays a critical role in the de novo synthesis of infectious virus following transfection of viral DNA. *J Virol* **63**(11)**:** 4579-4589

Chee AV, Lopez P, Pandolfi PP, Roizman B (2003) Promyelocytic leukemia protein mediates interferon-based anti-herpes simplex virus 1 effects. *J Virol* **77**(12)**:** 7101-7105

Chen J, Silverstein S (1992) Herpes simplex viruses with mutations in the gene encoding ICP0 are defective in gene expression. *J Virol* **66**(5)**:** 2916-2927

Cheung P, Panning B, Smiley JR (1997) Herpes simplex virus immediate-early proteins ICP0 and ICP4 activate the endogenous human alpha-globin gene in nonerythroid cells. *J Virol* **71**(3)**:** 1784-1793

Chou J, Kern ER, Whitley RJ, Roizman B (1990) Mapping of herpes simplex virus-1 neurovirulence to gamma 134.5, a gene nonessential for growth in culture. *Science* **250**(4985)**:** 1262-1266

Chou J, Roizman B (1992) The gamma 1(34.5) gene of herpes simplex virus 1 precludes neuroblastoma cells from triggering total shutoff of protein synthesis characteristic of programed cell death in neuronal cells. *Proc Natl Acad Sci U S A* **89**(8)**:** 3266-3270

Connolly SA, Jackson JO, Jardetzky TS, Longnecker R (2011) Fusing structure and function: a structural view of the herpesvirus entry machinery. *Nat Rev Microbiol* **9**(5)**:** 369-381

Corti O, Sabate O, Horellou P, Colin P, Dumas S, Buchet D, Buc-Caron MH, Mallet J (1999) A single adenovirus vector mediates doxycycline-controlled expression of tyrosine hydroxylase in brain grafts of human neural progenitors. *Nat Biotechnol* **17**(4)**:** 349-354

Cunningham C, Davison AJ (1993) A cosmid-based system for constructing mutants of herpes simplex virus type 1. *Virology* **197**(1)**:** 116-124

David C. Bloom DLK (2011) HSV-1 latency and the roles of the LATs. In *Alphaherpesviruses*, Weller SK (ed), 17, pp 295-315. Caister academic press

Davido DJ, Leib DA (1998) Analysis of the basal and inducible activities of the ICPO promoter of herpes simplex virus type 1. *J Gen Virol* **79 (Pt 9):** 2093-2098

Davison AJ, Wilkie NM (1981) Nucleotide sequences of the joint between the L and S segments of herpes simplex virus types 1 and 2. *J Gen Virol* **55**(Pt 2)**:** 315-331

DeLuca NA, McCarthy AM, Schaffer PA (1985) Isolation and characterization of deletion mutants of herpes simplex virus type 1 in the gene encoding immediate-early regulatory protein ICP4. *J Virol* **56**(2)**:** 558-570

Deuschle U, Meyer WK, Thiesen HJ (1995) Tetracycline-reversible silencing of eukaryotic promoters. *Mol Cell Biol* **15**(4)**:** 1907-1914

Dixon RA, Schaffer PA (1980) Fine-structure mapping and functional analysis of temperature-sensitive mutants in the gene encoding the herpes simplex virus type 1 immediate early protein VP175. *J Virol* **36**(1)**:** 189-203

Douville P, Hagmann M, Georgiev O, Schaffner W (1995) Positive and negative regulation at the herpes simplex virus ICP4 and ICP0 TAATGARAT motifs. *Virology* **207**(1)**:** 107-116

Durfee T, Nelson R, Baldwin S, Plunkett G, 3rd, Burland V, Mau B, Petrosino JF, Qin X, Muzny DM, Ayele M, Gibbs RA, Csorgo B, Posfai G, Weinstock GM, Blattner FR (2008) The complete genome sequence of Escherichia coli DH10B: insights into the biology of a laboratory workhorse. *J Bacteriol* **190**(7)**:** 2597-2606

Earley EM JK (1988) The lineage of Vero, Vero 76 and its clone C1008 in the United States, Vero cells: origin, properties and biomedical applicationsTokyoChiba Univ.**:** pp. 26-29

Efstathiou S, Preston CM (2005) Towards an understanding of the molecular basis of herpes simplex virus latency. *Virus Res* **111**(2)**:** 108-119

Ejercito PM, Kieff ED, Roizman B (1968) Characterization of herpes simplex virus strains differing in their effects on social behaviour of infected cells. *J Gen Virol* **2**(3)**:** 357-364

Everett RD (1984) Trans activation of transcription by herpes virus products: requirement for two HSV-1 immediate-early polypeptides for maximum activity. *EMBO J* **3**(13)**:** 3135-3141

Everett RD (1985) Activation of cellular promoters during herpes virus infection of biochemically transformed cells. *EMBO J* **4**(8)**:** 1973-1980

Everett RD (1989) Construction and characterization of herpes simplex virus type 1 mutants with defined lesions in immediate early gene 1. *J Gen Virol* **70 (Pt 5):** 1185-1202

Everett RD (2000) ICP0, a regulator of herpes simplex virus during lytic and latent infection. *Bioessays* **22**(8)**:** 761-770

Everett RD (2001) DNA viruses and viral proteins that interact with PML nuclear bodies. *Oncogene* **20**(49)**:** 7266-7273

Everett RD (2004) Herpes simplex virus type 1 regulatory protein ICP0 does not protect cyclins D1 and D3 from degradation during infection. *J Virol* **78**(18)**:** 9599-9604

Everett RD, Boutell C, McNair C, Grant L, Orr A (2010) Comparison of the biological and biochemical activities of several members of the alphaherpesvirus ICP0 family of proteins. *J Virol* **84**(7)**:** 3476-3487

Everett RD, Boutell C, Orr A (2004) Phenotype of a herpes simplex virus type 1 mutant that fails to express immediate-early regulatory protein ICP0. *J Virol* **78**(4)**:** 1763-1774

Everett RD, Chelbi-Alix MK (2007) PML and PML nuclear bodies: implications in antiviral defence. *Biochimie* **89**(6-7)**:** 819-830

Everett RD, Freemont P, Saitoh H, Dasso M, Orr A, Kathoria M, Parkinson J (1998) The disruption of ND10 during herpes simplex virus infection correlates with the Vmw110- and proteasome-dependent loss of several PML isoforms. *J Virol* **72**(8)**:** 6581-6591

Everett RD, Maul GG (1994) HSV-1 IE protein Vmw110 causes redistribution of PML. *EMBO J* **13**(21)**:** 5062-5069

Everett RD, Murray J (2005) ND10 components relocate to sites associated with herpes simplex virus type 1 nucleoprotein complexes during virus infection. *J Virol* **79**(8)**:** 5078-5089

Everett RD, Parada C, Gripon P, Sirma H, Orr A (2008) Replication of ICP0-null mutant herpes simplex virus type 1 is restricted by both PML and Sp100. *J Virol* **82**(6)**:** 2661-2672

Everett RD, Rechter S, Papior P, Tavalai N, Stamminger T, Orr A (2006) PML contributes to a cellular mechanism of repression of herpes simplex virus type 1 infection that is inactivated by ICP0. *J Virol* **80**(16)**:** 7995-8005

Everts B, van der Poel HG (2005) Replication-selective oncolytic viruses in the treatment of cancer. *Cancer Gene Ther* **12**(2)**:** 141-161

Faber SW, Wilcox KW (1986) Association of the herpes simplex virus regulatory protein ICP4 with specific nucleotide sequences in DNA. *Nucleic Acids Res* **14**(15)**:** 6067-6083

Ferenczy MW, Ranayhossaini DJ, Deluca NA (2011) Activities of ICP0 involved in the reversal of silencing of quiescent herpes simplex virus 1. *J Virol* **85**(10)**:** 4993-5002

Fink DJ, DeLuca NA, Yamada M, Wolfe DP, Glorioso JC (2000) Design and application of HSV vectors for neuroprotection. *Gene Ther* **7**(2)**:** 115-119

Fotaki ME, Pink JR, Mous J (1997) Tetracycline-responsive gene expression in mouse brain after amplicon-mediated gene transfer. *Gene Ther* **4**(9)**:** 901-908

Frampton AR, Jr., Goins WF, Nakano K, Burton EA, Glorioso JC (2005) HSV trafficking and development of gene therapy vectors with applications in the nervous system. *Gene Ther* **12**(11)**:** 891-901

Freundlieb S, Schirra-Muller C, Bujard H (1999) A tetracycline controlled activation/repression system with increased potential for gene transfer into mammalian cells. *J Gene Med* **1**(1)**:** 4-12

Fuller AO, Lee WC (1992) Herpes simplex virus type 1 entry through a cascade of virus-cell interactions requires different roles of gD and gH in penetration. *J Virol* **66**(8)**:** 5002-5012

Gaffney DF, McLauchlan J, Whitton JL, Clements JB (1985) A modular system for the assay of transcription regulatory signals: the sequence TAATGARAT is required for herpes simplex virus immediate early gene activation. *Nucleic Acids Res* **13**(21)**:** 7847-7863

Gao Q, Sun M, Wang X, Zhang GR, Geller AI (2006) Long-term inducible expression in striatal neurons from helper virus-free HSV-1 vectors that contain the tetracycline-inducible promoter system. *Brain Res* **1083**(1)**:** 1-13

Gatz C, Frohberg C, Wendenburg R (1992) Stringent repression and homogeneous de-repression by tetracycline of a modified CaMV 35S promoter in intact transgenic tobacco plants. *Plant J* **2**(3)**:** 397-404

Gatz C, Quail PH (1988) Tn10-encoded tet repressor can regulate an operator-containing plant promoter. *Proc Natl Acad Sci U S A* **85**(5)**:** 1394-1397

Geller AI, Keyomarsi K, Bryan J, Pardee AB (1990) An efficient deletion mutant packaging system for defective herpes simplex virus vectors: potential applications to human gene therapy and neuronal physiology. *Proc Natl Acad Sci U S A* **87**(22)**:** 8950-8954

Gelman IH, Silverstein S (1985) Identification of immediate early genes from herpes simplex virus that transactivate the virus thymidine kinase gene. *Proc Natl Acad Sci U S A* **82**(16)**:** 5265-5269

Gelman IH, Silverstein S (1986) Co-ordinate regulation of herpes simplex virus gene expression is mediated by the functional interaction of two immediate early gene products. *J Mol Biol* **191**(3)**:** 395-409

Gerster T, Roeder RG (1988) A herpesvirus trans-activating protein interacts with transcription factor OTF-1 and other cellular proteins. *Proc Natl Acad Sci U S A* **85**(17)**:** 6347-6351

Gierasch WW, Zimmerman DL, Ward SL, Vanheyningen TK, Romine JD, Leib DA (2006) Construction and characterization of bacterial artificial chromosomes containing HSV-1 strains 17 and KOS. *J Virol Methods* **135**(2)**:** 197-206

Glorioso JC (2014) Herpes simplex viral vectors: late bloomers with big potential. *Hum Gene Ther* **25**(2)**:** 83-91

Gossen M, Bonin AL, Bujard H (1993) Control of gene activity in higher eukaryotic cells by prokaryotic regulatory elements. *Trends Biochem Sci* **18**(12)**:** 471-475

Gossen M, Bujard H (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc Natl Acad Sci U S A* **89**(12)**:** 5547-5551

Gossen M, Freundlieb S, Bender G, Muller G, Hillen W, Bujard H (1995) Transcriptional activation by tetracyclines in mammalian cells. *Science* **268**(5218)**:** 1766-1769

Goverdhana S, Puntel M, Xiong W, Zirger JM, Barcia C, Curtin JF, Soffer EB, Mondkar S, King GD, Hu J, Sciascia SA, Candolfi M, Greengold DS, Lowenstein PR, Castro MG (2005) Regulatable gene expression systems for gene therapy applications: progress and future challenges. *Mol Ther* **12**(2)**:** 189-211

Grant SG, Jessee J, Bloom FR, Hanahan D (1990) Differential plasmid rescue from transgenic mouse DNAs into Escherichia coli methylation-restriction mutants. *Proc Natl Acad Sci U S A* **87**(12)**:** 4645-4649

Gu H, Poon AP, Roizman B (2009) During its nuclear phase the multifunctional regulatory protein ICP0 undergoes proteolytic cleavage characteristic of polyproteins. *Proc Natl Acad Sci U S A* **106**(45)**:** 19132-19137

Haberman RP, McCown TJ, Samulski RJ (1998) Inducible long-term gene expression in brain with adeno-associated virus gene transfer. *Gene Ther* **5**(12)**:** 1604-1611

Hagglund R, Roizman B (2002) Characterization of the novel E3 ubiquitin ligase encoded in exon 3 of herpes simplex virus-1-infected cell protein 0. *Proc Natl Acad Sci U S A* **99**(12)**:** 7889-7894

Hagglund R, Roizman B (2003) Herpes simplex virus 1 mutant in which the ICP0 HUL-1 E3 ubiquitin ligase site is disrupted stabilizes cdc34 but degrades D-type cyclins and exhibits diminished neurotoxicity. *J Virol* **77**(24)**:** 13194-13202

Hagglund R, Roizman B (2004) Role of ICP0 in the strategy of conquest of the host cell by herpes simplex virus 1. *J Virol* **78**(5)**:** 2169-2178

Hagglund R, Van Sant C, Lopez P, Roizman B (2002) Herpes simplex virus 1-infected cell protein 0 contains two E3 ubiquitin ligase sites specific for different E2 ubiquitin-conjugating enzymes. *Proc Natl Acad Sci U S A* **99**(2)**:** 631-636

Hagmann M, Georgiev O, Schaffner W (1997) The VP16 paradox: herpes simplex virus VP16 contains a long-range activation domain but within the natural multiprotein complex activates only from promoter-proximal positions. *J Virol* **71**(8)**:** 5952-5962

Hanahan D (1983) Studies on transformation of Escherichia coli with plasmids. *J Mol Biol* **166**(4)**:** 557-580

Harle P, Sainz B, Jr., Carr DJ, Halford WP (2002) The immediate-early protein, ICP0, is essential for the resistance of herpes simplex virus to interferon-alpha/beta. *Virology* **293**(2)**:** 295-304

Harmenberg J, Wahren B (1982) Influence of cell culture conditions on the inhibition of herpes simplex virus type 1 replication by acyclovir. *Intervirology* **17**(4)**:** 215-221

Harris RA, Everett RD, Zhu XX, Silverstein S, Preston CM (1989) Herpes simplex virus type 1 immediate-early protein Vmw110 reactivates latent herpes simplex virus type 2 in an in vitro latency system. *J Virol* **63**(8)**:** 3513-3515

Herold BC, Visalli RJ, Susmarski N, Brandt CR, Spear PG (1994) Glycoprotein C-independent binding of herpes simplex virus to cells requires cell surface heparan sulphate and glycoprotein B. *J Gen Virol* **75 (Pt 6):** 1211-1222

Herrlinger U, Pechan PA, Jacobs AH, Woiciechowski C, Rainov NG, Fraefel C, Paulus W, Reeves SA (2000) HSV-1 infected cell proteins influence tetracycline-regulated transgene expression. *J Gene Med* **2**(5)**:** 379-389

Ho DY, McLaughlin JR, Sapolsky RM (1996) Inducible gene expression from defective herpes simplex virus vectors using the tetracycline-responsive promoter system. *Brain Res Mol Brain Res* **41**(1-2)**:** 200-209

Hobbs S, Jitrapakdee S, Wallace JC (1998) Development of a bicistronic vector driven by the human polypeptide chain elongation factor 1alpha promoter for creation of stable mammalian cell lines that express very high levels of recombinant proteins. *Biochem Biophys Res Commun* **252**(2)**:** 368-372

Hofmann A, Nolan GP, Blau HM (1996) Rapid retroviral delivery of tetracycline-inducible genes in a single autoregulatory cassette. *Proc Natl Acad Sci U S A* **93**(11)**:** 5185-5190

Homa FL, Glorioso JC, Levine M (1988) A specific 15-bp TATA box promoter element is required for expression of a herpes simplex virus type 1 late gene. *Genes Dev* **2**(1)**:** 40-53

Honess RW, Roizman B (1974) Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. *J Virol* **14**(1)**:** 8-19

Hynes NE, Kennedy N, Rahmsdorf U, Groner B (1981) Hormone-responsive expression of an endogenous proviral gene of mouse mammary tumor virus after molecular cloning and gene transfer into cultured cells. *Proc Natl Acad Sci U S A* **78**(4)**:** 2038-2042

Israel DI, Kaufman RJ (1989) Highly inducible expression from vectors containing multiple GRE's in CHO cells overexpressing the glucocorticoid receptor. *Nucleic Acids Res* **17**(12)**:** 4589-4604

Itzhaki RF, Wozniak MA (2008) Herpes simplex virus type 1 in Alzheimer's disease: the enemy within. *J Alzheimers Dis* **13**(4)**:** 393-405

Jamieson DR, Robinson LH, Daksis JI, Nicholl MJ, Preston CM (1995) Quiescent viral genomes in human fibroblasts after infection with herpes simplex virus type 1 Vmw65 mutants. *J Gen Virol* **76 (Pt 6):** 1417-1431

Jenkins FJ, Donoghue AM, Martin JR (1996) Deletion of the Herpes simplex 1 internal repeat sequences affects pathogenicity in the mouse. *Front Biosci* **1:** a59-68

Jenkins FJ, Martin JR (1990) Role of the herpes simplex virus 1 internal repeat sequences in pathogenicity. *Intervirology* **31**(2-4)**:** 129-138

Jenkins FJ, Roizman B (1986) Herpes simplex virus 1 recombinants with noninverting genomes frozen in different isomeric arrangements are capable of independent replication. *J Virol* **59**(2)**:** 494-499

Jiang Y, Wei N, Zhu J, Zhai D, Wu L, Chen M, Xu G, Liu X (2012) A new approach with less damage: intranasal delivery of tetracycline-inducible replication-defective herpes simplex virus type-1 vector to brain. *Neuroscience* **201:** 96-104

Junejo F, Brown SM (1995) Deletions and duplication in internal inverted repeat sequence of long region/unique sequence of long region (IRL/UL) of herpes simplex virus type-1 (HSV-1) genome are not

evidently associated with intracranial and foot-pad pathogenicity in mouse model. *J Pak Med Assoc* **45**(4)**:** 95-98

Kalamvoki M, Roizman B (2010) Role of herpes simplex virus ICP0 in the transactivation of genes introduced by infection or transfection: a reappraisal. *J Virol* **84**(9)**:** 4222-4228

Kawaguchi Y, Bruni R, Roizman B (1997) Interaction of herpes simplex virus 1 alpha regulatory protein ICP0 with elongation factor 1delta: ICP0 affects translational machinery. *J Virol* **71**(2)**:** 1019-1024

Keeling PJ, Palmer JD (2008) Horizontal gene transfer in eukaryotic evolution. *Nat Rev Genet* **9**(8)**:** 605-618

Kemp LM, Dent CL, Latchman DS (1990) Octamer motif mediates transcriptional repression of HSV immediate-early genes and octamer-containing cellular promoters in neuronal cells. *Neuron* **4**(2)**:** 215-222

Kesari S, Lasner TM, Balsara KR, Randazzo BP, Lee VM, Trojanowski JQ, Fraser NW (1998) A neuroattenuated ICP34.5-deficient herpes simplex virus type 1 replicates in ependymal cells of the murine central nervous system. *J Gen Virol* **79 (Pt 3):** 525-536

Kim HJ, Gatz C, Hillen W, Jones TR (1995) Tetracycline repressor-regulated gene repression in recombinant human cytomegalovirus. *J Virol* **69**(4)**:** 2565-2573

Kim SY, Horrigan SK, Altenhofen JL, Arbieva ZH, Hoffman R, Westbrook CA (1998) Modification of bacterial artificial chromosome clones using Cre recombinase: introduction of selectable markers for expression in eukaryotic cells. *Genome Res* **8**(4)**:** 404-412

Klemm RD, Goodrich JA, Zhou S, Tjian R (1995) Molecular cloning and expression of the 32-kDa subunit of human TFIID reveals interactions with VP16 and TFIIB that mediate transcriptional activation. *Proc Natl Acad Sci U S A* **92**(13)**:** 5788-5792

Koyama AH, Uchida T (1987) The mode of entry of herpes simplex virus type 1 into Vero cells. *Microbiol Immunol* **31**(2)**:** 123-130

Kristensson K, Lycke E, Sjostrand J (1971) Spread of herpes simplex virus in peripheral nerves. *Acta Neuropathol* **17**(1)**:** 44-53

Kristie TM, Roizman B (1987) Host cell proteins bind to the cis-acting site required for virion-mediated induction of herpes simplex virus 1 alpha genes. *Proc Natl Acad Sci U S A* **84**(1)**:** 71-75

Kwun HJ, Jang KL (2000) Transcriptional regulation of herpes simplex virus type 1 ICP0 promoter by virion protein 16. *Mol Cell Biol Res Commun* **3**(1)**:** 15-19

Lai JS, Herr W (1997) Interdigitated residues within a small region of VP16 interact with Oct-1, HCF, and DNA. *Mol Cell Biol* **17**(7)**:** 3937-3946

Lamartina S, Silvi L, Roscilli G, Casimiro D, Simon AJ, Davies ME, Shiver JW, Rinaudo CD, Zampaglione I, Fattori E, Colloca S, Gonzalez Paz O, Laufer R, Bujard H, Cortese R, Ciliberto G, Toniatti C (2003) Construction of an rtTA2(s)-m2/tts(kid)-based transcription regulatory switch that displays no basal activity, good inducibility, and high responsiveness to doxycycline in mice and non-human primates. *Mol Ther* **7**(2)**:** 271-280

Lanfranca MP, Mostafa HH, Davido DJ (2014) HSV-1 ICP0: An E3 Ubiquitin Ligase That Counteracts Host Intrinsic and Innate Immunity. *Cells* **3**(2)**:** 438-454

Le Page C, Genin P, Baines MG, Hiscott J (2000) Interferon activation and innate immunity. *Rev Immunogenet* **2**(3)**:** 374-386

Lee F, Mulligan R, Berg P, Ringold G (1981) Glucocorticoids regulate expression of dihydrofolate reductase cDNA in mouse mammary tumour virus chimaeric plasmids. *Nature* **294**(5838)**:** 228-232

Leib DA, Harrison TE, Laslo KM, Machalek MA, Moorman NJ, Virgin HW (1999) Interferons regulate the phenotype of wild-type and mutant herpes simplex viruses in vivo. *J Exp Med* **189**(4)**:** 663-672

Li Y, Zhang C, Chen X, Yu J, Wang Y, Yang Y, Du M, Jin H, Ma Y, He B, Cao Y (2011) ICP34.5 protein of herpes simplex virus facilitates the initiation of protein translation by bridging eukaryotic initiation factor 2alpha (eIF2alpha) and protein phosphatase 1. *J Biol Chem* **286**(28)**:** 24785-24792

Liang Y, Vogel JL, Narayanan A, Peng H, Kristie TM (2009) Inhibition of the histone demethylase LSD1 blocks alpha-herpesvirus lytic replication and reactivation from latency. *Nat Med* **15**(11)**:** 1312-1317

Liesegang TJ (1992) Biology and molecular aspects of herpes simplex and varicella-zoster virus infections. *Ophthalmology* **99**(5)**:** 781-799

Liesegang TJ (2001) Herpes simplex virus epidemiology and ocular importance. *Cornea* **20**(1)**:** 1-13

Lilley CE, Coffin RS (2003) Construction of multiply disabled herpes simplex viral vectors for gene delivery to the nervous system. *Methods Mol Med* **76:** 33-49

Lim F (2013) HSV-1 as a model for emerging gene delivery vehicles. *ISRN Virology*

Lin R, Noyce RS, Collins SE, Everett RD, Mossman KL (2004) The herpes simplex virus ICP0 RING finger domain inhibits IRF3- and IRF7-mediated activation of interferon-stimulated genes. *J Virol* **78**(4)**:** 1675-1684

Liu M, Rakowski B, Gershburg E, Weisend CM, Lucas O, Schmidt EE, Halford WP (2010) ICP0 antagonizes ICP4-dependent silencing of the herpes simplex virus ICP0 gene. *PLoS One* **5**(1)**:** e8837

Liu Y, Gong W, Huang CC, Herr W, Cheng X (1999) Crystal structure of the conserved core of the herpes simplex virus transcriptional regulatory protein VP16. *Genes Dev* **13**(13)**:** 1692-1703

Lium EK, Panagiotidis CA, Wen X, Silverstein S (1996) Repression of the alpha0 gene by ICP4 during a productive herpes simplex virus infection. *J Virol* **70**(6)**:** 3488-3496

Longnecker R, Roizman B (1986) Generation of an inverting herpes simplex virus 1 mutant lacking the L-S junction a sequences, an origin of DNA synthesis, and several genes including those specifying glycoprotein E and the alpha 47 gene. *J Virol* **58**(2)**:** 583-591

Lopez P, Van Sant C, Roizman B (2001) Requirements for the nuclear-cytoplasmic translocation of infected-cell protein 0 of herpes simplex virus 1. *J Virol* **75**(8)**:** 3832-3840

Lowenstein A (1919) Aetiologische untersuchungen uber den fieber- haften, herpes. Munch Med Wochenschr. **66:** 769-770.

Lukashchuk V, Everett RD (2010) Regulation of ICP0-null mutant herpes simplex virus type 1 infection by ND10 components ATRX and hDaxx. *J Virol* **84**(8)**:** 4026-4040

Macdonald SJ, Mostafa HH, Morrison LA, Davido DJ (2012a) Genome sequence of herpes simplex virus 1 strain KOS. *J Virol* **86**(11)**:** 6371-6372

Macdonald SJ, Mostafa HH, Morrison LA, Davido DJ (2012b) Genome sequence of herpes simplex virus 1 strain McKrae. *J Virol* **86**(17)**:** 9540-9541

Mak AB, Ni Z, Hewel JA, Chen GI, Zhong G, Karamboulas K, Blakely K, Smiley S, Marcon E, Roudeva D, Li J, Olsen JB, Wan C, Punna T, Isserlin R, Chetyrkin S, Gingras AC, Emili A, Greenblatt J, Moffat J (2010) A lentiviral functional proteomics approach identifies chromatin remodeling complexes important for the induction of pluripotency. *Mol Cell Proteomics* **9**(5)**:** 811-823

Mamane Y, Heylbroeck C, Genin P, Algarte M, Servant MJ, LePage C, DeLuca C, Kwon H, Lin R, Hiscott J (1999) Interferon regulatory factors: the next generation. *Gene* **237**(1)**:** 1-14

Marconi P, Manservigi R, Epstein AL (2010) HSV-1-derived helper-independent defective vectors, replicating vectors and amplicon vectors, for the treatment of brain diseases. *Curr Opin Drug Discov Devel* **13**(2)**:** 169-183

Margolin JF, Friedman JR, Meyer WK, Vissing H, Thiesen HJ, Rauscher FJ, 3rd (1994) Kruppel-associated boxes are potent transcriptional repression domains. *Proc Natl Acad Sci U S A* **91**(10)**:** 4509-4513

Marzio G, Verhoef K, Vink M, Berkhout B (2001) In vitro evolution of a highly replicating, doxycycline-dependent HIV for applications in vaccine studies. *Proc Natl Acad Sci U S A* **98**(11)**:** 6342-6347

Mavromara-Nazos P, Silver S, Hubenthal-Voss J, McKnight JL, Roizman B (1986) Regulation of herpes simplex virus 1 genes: alpha gene sequence requirements for transient induction of indicator genes regulated by beta or late (gamma 2) promoters. *Virology* **149**(2)**:** 152-164

Mayo KE, Warren R, Palmiter RD (1982) The mouse metallothionein-I gene is transcriptionally regulated by cadmium following transfection into human or mouse cells. *Cell* **29**(1)**:** 99-108

McGeoch DJ, Dalrymple MA, Davison AJ, Dolan A, Frame MC, McNab D, Perry LJ, Scott JE, Taylor P (1988) The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. *J Gen Virol* **69 (Pt 7):** 1531-1574

McKnight JL, Kristie TM, Roizman B (1987) Binding of the virion protein mediating alpha gene induction in herpes simplex virus 1-infected cells to its cis site requires cellular proteins. *Proc Natl Acad Sci U S A* **84**(20)**:** 7061-7065

Mocarski ES, Roizman B (1982) Structure and role of the herpes simplex virus DNA termini in inversion, circularization and generation of virion DNA. *Cell* **31**(1)**:** 89-97

Montgomery RI, Warner MS, Lum BJ, Spear PG (1996) Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. *Cell* **87**(3)**:** 427-436

Moosmann P, Georgiev O, Thiesen HJ, Hagmann M, Schaffner W (1997) Silencing of RNA polymerases II and III-dependent transcription by the KRAB protein domain of KOX1, a Kruppel-type zinc finger factor. *Biol Chem* **378**(7)**:** 669-677

Mossman KL, Saffran HA, Smiley JR (2000) Herpes simplex virus ICP0 mutants are hypersensitive to interferon. *J Virol* **74**(4)**:** 2052-2056

Mossman KL, Smiley JR (2002) Herpes simplex virus ICP0 and ICP34.5 counteract distinct interferon-induced barriers to virus replication. *J Virol* **76**(4)**:** 1995-1998

Muller S, Dejean A (1999) Viral immediate-early proteins abrogate the modification by SUMO-1 of PML and Sp100 proteins, correlating with nuclear body disruption. *J Virol* **73**(6)**:** 5137-5143

Muyrers JP, Zhang Y, Stewart AF (2001) Techniques: Recombinogenic engineering--new options for cloning and manipulating DNA. *Trends Biochem Sci* **26**(5)**:** 325-331

Muyrers JP, Zhang Y, Testa G, Stewart AF (1999) Rapid modification of bacterial artificial chromosomes by ET-recombination. *Nucleic Acids Res* **27**(6)**:** 1555-1557

Nicola AV, McEvoy AM, Straus SE (2003) Roles for endocytosis and low pH in herpes simplex virus entry into HeLa and Chinese hamster ovary cells. *J Virol* **77**(9)**:** 5324-5332

Nishiyama Y (1996) Herpesvirus genes: molecular basis of viral replication and pathogenicity. *Nagoya J Med Sci* **59**(3-4)**:** 107-119

Norberg P, Tyler S, Severini A, Whitley R, Liljeqvist JA, Bergstrom T (2011) A genome-wide comparative evolutionary analysis of herpes simplex virus type 1 and varicella zoster virus. *PLoS One* **6**(7)**:** e22527

O'Hare P, Goding CR, Haigh A (1988) Direct combinatorial interaction between a herpes simplex virus regulatory protein and a cellular octamer-binding factor mediates specific induction of virus immediate-early gene expression. *EMBO J* **7**(13)**:** 4231-4238

O'Hare P, Hayward GS (1985) Evidence for a direct role for both the 175,000- and 110,000-molecular-weight immediate-early proteins of herpes simplex virus in the transactivation of delayed-early promoters. *J Virol* **53**(3)**:** 751-760

Pan D, Flores O, Umbach JL, Pesola JM, Bentley P, Rosato PC, Leib DA, Cullen BR, Coen DM (2014) A neuron-specific host microRNA targets herpes simplex virus-1 ICP0 expression and promotes latency. *Cell Host Microbe* **15**(4)**:** 446-456

Perez MC, Hunt SP, Coffin RS, Palmer JA (2004) Comparative analysis of genomic HSV vectors for gene delivery to motor neurons following peripheral inoculation in vivo. *Gene Ther* **11**(13)**:** 1023-1032

Perry LJ, Rixon FJ, Everett RD, Frame MC, McGeoch DJ (1986) Characterization of the IE110 gene of herpes simplex virus type 1. *J Gen Virol* **67 (Pt 11):** 2365-2380

Pestka S, Langer JA, Zoon KC, Samuel CE (1987) Interferons and their actions. *Annu Rev Biochem* **56:** 727-777

Poffenberger KL, Raichlen PE, Herman RC (1993) In vitro characterization of a herpes simplex virus type 1 ICP22 deletion mutant. *Virus Genes* **7**(2)**:** 171-186

Poffenberger KL, Tabares E, Roizman B (1983) Characterization of a viable, noninverting herpes simplex virus 1 genome derived by insertion and deletion of sequences at the junction of components L and S. *Proc Natl Acad Sci U S A* **80**(9)**:** 2690-2694

Post LE, Roizman B (1981) A generalized technique for deletion of specific genes in large genomes: alpha gene 22 of herpes simplex virus 1 is not essential for growth. *Cell* **25**(1)**:** 227-232

Preston CM (1979) Abnormal properties of an immediate early polypeptide in cells infected with the herpes simplex virus type 1 mutant tsK. *J Virol* **32**(2)**:** 357-369

Preston CM, Mabbs R, Nicholl MJ (1997) Construction and characterization of herpes simplex virus type 1 mutants with conditional defects in immediate early gene expression. *Virology* **229**(1)**:** 228-239

Regad T, Chelbi-Alix MK (2001) Role and fate of PML nuclear bodies in response to interferon and viral infections. *Oncogene* **20**(49)**:** 7274-7286

Reich E, Franklin RM, Shatkin AJ, Tatum EL (1961) Effect of actinomycin D on cellular nucleic acid synthesis and virus production. *Science* **134**(3478)**:** 556-557

Reske A, Pollara G, Krummenacher C, Chain BM, Katz DR (2007) Understanding HSV-1 entry glycoproteins. *Rev Med Virol* **17**(3)**:** 205-215

Rice SA, Davido DJ (2013) HSV-1 ICP22: hijacking host nuclear functions to enhance viral infection. *Future Microbiol* **8**(3)**:** 311-321

Robbins PD, Tahara H, Ghivizzani SC (1998) Viral vectors for gene therapy. *Trends Biotechnol* **16**(1)**:** 35-40

Roizman B (1979a) The organization of the herpes simplex virus genomes. *Annu Rev Genet* **13:** 25-57

Roizman B (1979b) The structure and isomerization of herpes simplex virus genomes. *Cell* **16**(3)**:** 481-494

Roizman B (2011) The checkpoints of viral gene expression in productive and latent infection: the role of the HDAC/CoREST/LSD1/REST repressor complex. *J Virol* **85**(15)**:** 7474-7482

Roizman B, Whitley RJ (2013) An inquiry into the molecular basis of HSV latency and reactivation. *Annu Rev Microbiol* **67:** 355-374

Roizmann B, Desrosiers RC, Fleckenstein B, Lopez C, Minson AC, Studdert MJ (1992) The family Herpesviridae: an update. The Herpesvirus Study Group of the International Committee on Taxonomy of Viruses. *Arch Virol* **123**(3-4)**:** 425-449

Sacks WR, Greene CC, Aschman DP, Schaffer PA (1985) Herpes simplex virus type 1 ICP27 is an essential regulatory protein. *J Virol* **55**(3)**:** 796-805

Sacks WR, Schaffer PA (1987) Deletion mutants in the gene encoding the herpes simplex virus type 1 immediate-early protein ICP0 exhibit impaired growth in cell culture. *J Virol* **61**(3)**:** 829-839

Saeki Y, Fraefel C, Ichikawa T, Breakefield XO, Chiocca EA (2001) Improved helper virus-free packaging system for HSV amplicon vectors using an ICP27-deleted, oversized HSV-1 DNA in a bacterial artificial chromosome. *Mol Ther* **3**(4)**:** 591-601

Saeki Y, Ichikawa T, Saeki A, Chiocca EA, Tobler K, Ackermann M, Breakefield XO, Fraefel C (1998) Herpes simplex virus type 1 DNA amplified as bacterial artificial chromosome in Escherichia coli: rescue of replication-competent virus progeny and packaging of amplicon vectors. *Hum Gene Ther* **9**(18)**:** 2787-2794

Sauer A, Wang JB, Hahn G, McVoy MA (2010) A human cytomegalovirus deleted of internal repeats replicates with near wild type efficiency but fails to undergo genome isomerization. *Virology* **401**(1)**:** 90-95

Sawtell NM, Thompson RL (1992) Rapid in vivo reactivation of herpes simplex virus in latently infected murine ganglionic neurons after transient hyperthermia. *J Virol* **66**(4)**:** 2150-2156

Schmeisser F, Donohue M, Weir JP (2002) Tetracycline-regulated gene expression in replication-incompetent herpes simplex virus vectors. *Hum Gene Ther* **13**(18)**:** 2113-2124

Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* **9**(7)**:** 671-675

Searle PF, Stuart GW, Palmiter RD (1985) Building a metal-responsive promoter with synthetic regulatory elements. *Mol Cell Biol* **5**(6)**:** 1480-1489

Sears AE, Hukkanen V, Labow MA, Levine AJ, Roizman B (1991) Expression of the herpes simplex virus 1 alpha transinducing factor (VP16) does not induce reactivation of latent virus or prevent the establishment of latency in mice. *J Virol* **65**(6)**:** 2929-2935

Sen GC (2001) Viruses and interferons. *Annu Rev Microbiol* **55:** 255-281

Senatore B, Cafieri A, Di Marino I, Rosati M, Di Nocera PP, Grimaldi G (1999) A variety of RNA polymerases II and III-dependent promoter classes is repressed by factors containing the Kruppel-associated/finger preceding box of zinc finger proteins. *Gene* **234**(2)**:** 381-394

Shi YJ, Matson C, Lan F, Iwase S, Baba T, Shi Y (2005) Regulation of LSD1 histone demethylase activity by its associated factors. *Mol Cell* **19**(6)**:** 857-864

Shockett P, Difilippantonio M, Hellman N, Schatz DG (1995) A modified tetracycline-regulated system provides autoregulatory, inducible gene expression in cultured cells and transgenic mice. *Proc Natl Acad Sci U S A* **92**(14)**:** 6522-6526

Shukla D, Liu J, Blaiklock P, Shworak NW, Bai X, Esko JD, Cohen GH, Eisenberg RJ, Rosenberg RD, Spear PG (1999) A novel role for 3-O-sulfated heparan sulfate in herpes simplex virus 1 entry. *Cell* **99**(1)**:** 13-22

Smith MC, Boutell C, Davido DJ (2011) HSV-1 ICP0: paving the way for viral replication. *Future Virol* **6**(4)**:** 421-429

Spaete RR, Frenkel N (1982) The herpes simplex virus amplicon: a new eucaryotic defective-virus cloning-amplifying vector. *Cell* **30**(1)**:** 295-304

Spear PG, Eisenberg RJ, Cohen GH (2000) Three classes of cell surface receptors for alphaherpesvirus entry. *Virology* **275**(1)**:** 1-8

Spear PG, Longnecker R (2003) Herpesvirus entry: an update. *J Virol* **77**(19)**:** 10179-10185

Steiner I, Spivack JG, Deshmane SL, Ace CI, Preston CM, Fraser NW (1990) A herpes simplex virus type 1 mutant containing a nontransinducing Vmw65 protein establishes latent infection in vivo in the absence of viral replication and reactivates efficiently from explanted trigeminal ganglia. *J Virol* **64**(4)**:** 1630-1638

Stevens JG, Wagner EK, Devi-Rao GB, Cook ML, Feldman LT (1987) RNA complementary to a herpesvirus alpha gene mRNA is prominent in latently infected neurons. *Science* **235**(4792)**:** 1056-1059

Stow EC, Stow ND (1989) Complementation of a herpes simplex virus type 1 Vmw110 deletion mutant by human cytomegalovirus. *J Gen Virol* **70 (Pt 3):** 695-704

Stow ND, Stow EC (1986) Isolation and characterization of a herpes simplex virus type 1 mutant containing a deletion within the gene encoding the immediate early polypeptide Vmw110. *J Gen Virol* **67 (Pt 12):** 2571-2585

Strang BL, Stow ND (2005) Circularization of the herpes simplex virus type 1 genome upon lytic infection. *J Virol* **79**(19)**:** 12487-12494

Strang BL, Stow ND (2007) Blocks to herpes simplex virus type 1 replication in a cell line, tsBN2, encoding a temperature-sensitive RCC1 protein. *J Gen Virol* **88**(Pt 2)**:** 376-383

Szpara ML, Parsons L, Enquist LW (2010) Sequence variability in clinical and laboratory isolates of herpes simplex virus 1 reveals new mutations. *J Virol* **84**(10)**:** 5303-5313

Szulc J, Wiznerowicz M, Sauvain MO, Trono D, Aebischer P (2006) A versatile tool for conditional gene expression and knockdown. *Nat Methods* **3**(2)**:** 109-116

Tanaka M, Kagawa H, Yamanashi Y, Sata T, Kawaguchi Y (2003) Construction of an excisable bacterial artificial chromosome containing a full-length infectious clone of herpes simplex virus type 1: viruses reconstituted from the clone exhibit wild-type properties in vitro and in vivo. *J Virol* **77**(2)**:** 1382-1391

Taylor RG, Walker DC, McInnes RR (1993) E. coli host strains significantly affect the quality of small scale plasmid DNA preparations used for sequencing. *Nucleic Acids Res* **21**(7)**:** 1677-1678

Thomas D, Blakqori G, Wagner V, Banholzer M, Kessler N, Elliott RM, Haller O, Weber F (2004) Inhibition of RNA polymerase II phosphorylation by a viral interferon antagonist. *J Biol Chem* **279**(30)**:** 31471-31477

Thomas S, Coffin RS, Watts P, Gough G, Latchman DS (1998) The TAATGARAT motif in the herpes simplex virus immediate-early gene promoters can confer both positive and negative responses to cellular octamer-binding proteins when it is located within the viral genome. *J Virol* **72**(4)**:** 3495-3500

Thompson RL, Preston CM, Sawtell NM (2009) De novo synthesis of VP16 coordinates the exit from HSV latency in vivo. *PLoS Pathog* **5**(3)**:** e1000352

Thompson RL, Sawtell NM (2006) Evidence that the herpes simplex virus type 1 ICP0 protein does not initiate reactivation from latency in vivo. *J Virol* **80**(22)**:** 10919-10930

Umene K, Nishimoto T (1996) Replication of herpes simplex virus type 1 DNA is inhibited in a temperature-sensitive mutant of BHK-21 cells lacking RCC1 (regulator of chromosome condensation) and virus DNA remains linear. *J Gen Virol* **77 (Pt 9):** 2261-2270

Urlinger S, Baron U, Thellmann M, Hasan MT, Bujard H, Hillen W (2000) Exploring the sequence space for tetracycline-dependent transcriptional activators: novel mutations yield expanded range and sensitivity. *Proc Natl Acad Sci U S A* **97**(14)**:** 7963-7968

Van Sant C, Hagglund R, Lopez P, Roizman B (2001) The infected cell protein 0 of herpes simplex virus 1 dynamically interacts with proteasomes, binds and activates the cdc34 E2 ubiquitin-conjugating enzyme, and possesses in vitro E3 ubiquitin ligase activity. *Proc Natl Acad Sci U S A* **98**(15)**:** 8815-8820

Varghese S, Rabkin SD (2002) Oncolytic herpes simplex virus vectors for cancer virotherapy. *Cancer Gene Ther* **9**(12)**:** 967-978

Verbruggen P, Ruf M, Blakqori G, Overby AK, Heidemann M, Eick D, Weber F (2011) Interferon antagonist NSs of La Crosse virus triggers a DNA damage response-like degradation of transcribing RNA polymerase II. *J Biol Chem* **286**(5)**:** 3681-3692

Verhoef K, Marzio G, Hillen W, Bujard H, Berkhout B (2001) Strict control of human immunodeficiency virus type 1 replication by a genetic switch: Tet for Tat. *J Virol* **75**(2)**:** 979-987

Wade-Martins R, Smith ER, Tyminski E, Chiocca EA, Saeki Y (2001) An infectious transfer and expression system for genomic DNA loci in human and mouse cells. *Nat Biotechnol* **19**(11)**:** 1067-1070

Wagner EK, Rice M, Sutherland BM (1975) Photoreactivation of herpes simplex virus in human fibroblasts. *Nature* **254**(5501)**:** 627-628

Wang S, Zhao Y, Leiby M, Zhu J (2009) A new positive/negative selection scheme for precise BAC recombineering. *Mol Biotechnol* **42**(1)**:** 110-116

Ward PL, Roizman B (1994) Herpes simplex genes: the blueprint of a successful human pathogen. *Trends Genet* **10**(8)**:** 267-274

Wathelet MG, Berr PM, Huez GA (1992) Regulation of gene expression by cytokines and virus in human cells lacking the type-I interferon locus. *Eur J Biochem* **206**(3)**:** 901-910

Watson RJ, Clements JB (1978) Characterization of transcription-deficient temperature-sensitive mutants of herpes simplex virus type 1. *Virology* **91**(2)**:** 364-379

Whitley RJ, Kimberlin DW, Roizman B (1998) Herpes simplex viruses. *Clin Infect Dis* **26**(3)**:** 541-553; quiz 554-545

Wiznerowicz M, Trono D (2003) Conditional suppression of cellular genes: lentivirus vector-mediated drug-inducible RNA interference. *J Virol* **77**(16)**:** 8957-8961

Wozniak MA, Frost AL, Preston CM, Itzhaki RF (2011) Antivirals reduce the formation of key Alzheimer's disease molecules in cell cultures acutely infected with herpes simplex virus type 1. *PLoS One* **6**(10)**:** e25152

Wurm FM, Gwinn KA, Kingston RE (1986) Inducible overproduction of the mouse c-myc protein in mammalian cells. *Proc Natl Acad Sci U S A* **83**(15)**:** 5414-5418

Wysocka J, Herr W (2003) The herpes simplex virus VP16-induced complex: the makings of a regulatory switch. *Trends Biochem Sci* **28**(6)**:** 294-304

Yang M, Gocke CB, Luo X, Borek D, Tomchick DR, Machius M, Otwinowski Z, Yu H (2006) Structural basis for CoREST-dependent demethylation of nucleosomes by the human LSD1 histone demethylase. *Mol Cell* **23**(3)**:** 377-387

Yao F, Murakami N, Bleiziffer O, Zhang P, Akhrameyeva NV, Xu X, Brans R (2010) Development of a regulatable oncolytic herpes simplex virus type 1 recombinant virus for tumor therapy. *J Virol* **84**(16)**:** 8163-8171

Yao F, Schaffer PA (1995) An activity specified by the osteosarcoma line U2OS can substitute functionally for ICP0, a major regulatory protein of herpes simplex virus type 1. *J Virol* **69**(10)**:** 6249-6258

Yao F, Svensjo T, Winkler T, Lu M, Eriksson C, Eriksson E (1998) Tetracycline repressor, tetR, rather than the tetR-mammalian cell transcription factor fusion derivatives, regulates inducible gene expression in mammalian cells. *Hum Gene Ther* **9**(13)**:** 1939-1950

Yao F, Theopold C, Hoeller D, Bleiziffer O, Lu Z (2006) Highly efficient regulation of gene expression by tetracycline in a replication-defective herpes simplex viral vector. *Mol Ther* **13**(6)**:** 1133-1141

Yao XD, Matecic M, Elias P (1997) Direct repeats of the herpes simplex virus a sequence promote nonconservative homologous recombination that is not dependent on XPF/ERCC4. *J Virol* **71**(9)**:** 6842-6849

Zhang Y, Buchholz F, Muyrers JP, Stewart AF (1998) A new logic for DNA engineering using recombination in Escherichia coli. *Nat Genet* **20**(2)**:** 123-128

Zilio N, Wehrkamp-Richter S, Boddy MN (2012) A new versatile system for rapid control of gene expression in the fission yeast Schizosaccharomyces pombe. *Yeast* **29**(10)**:** 425-434

Appendix